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BLOOD

The Journal of The American Society of Hematology

**American Society of Hematology
Twenty-Eighth Annual Meeting
December 6–9, 1986
San Francisco
(see page 982 for program summary)**

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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Please see brief summary of prescribing information on next page.

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Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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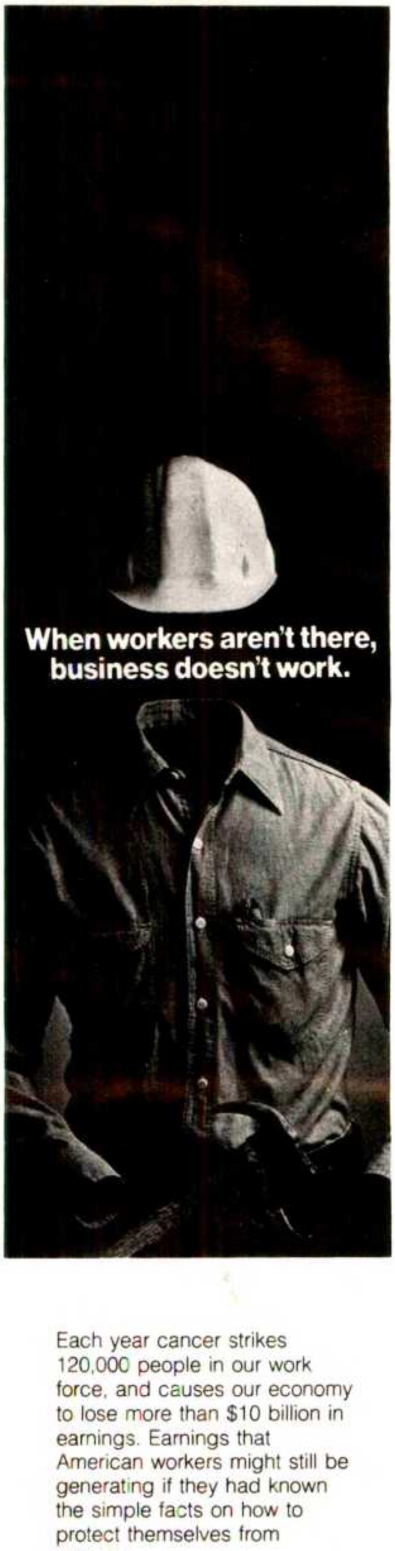

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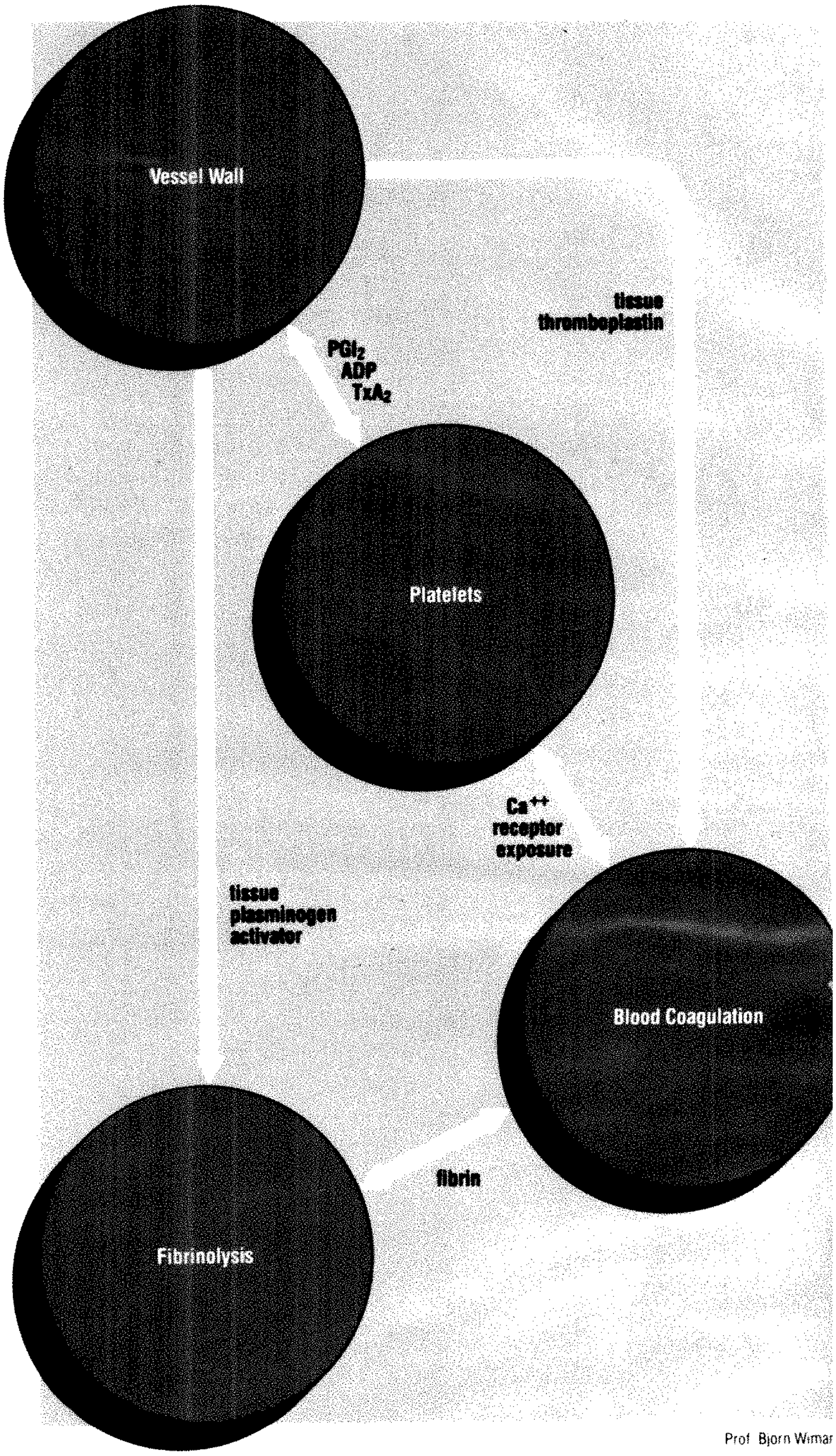

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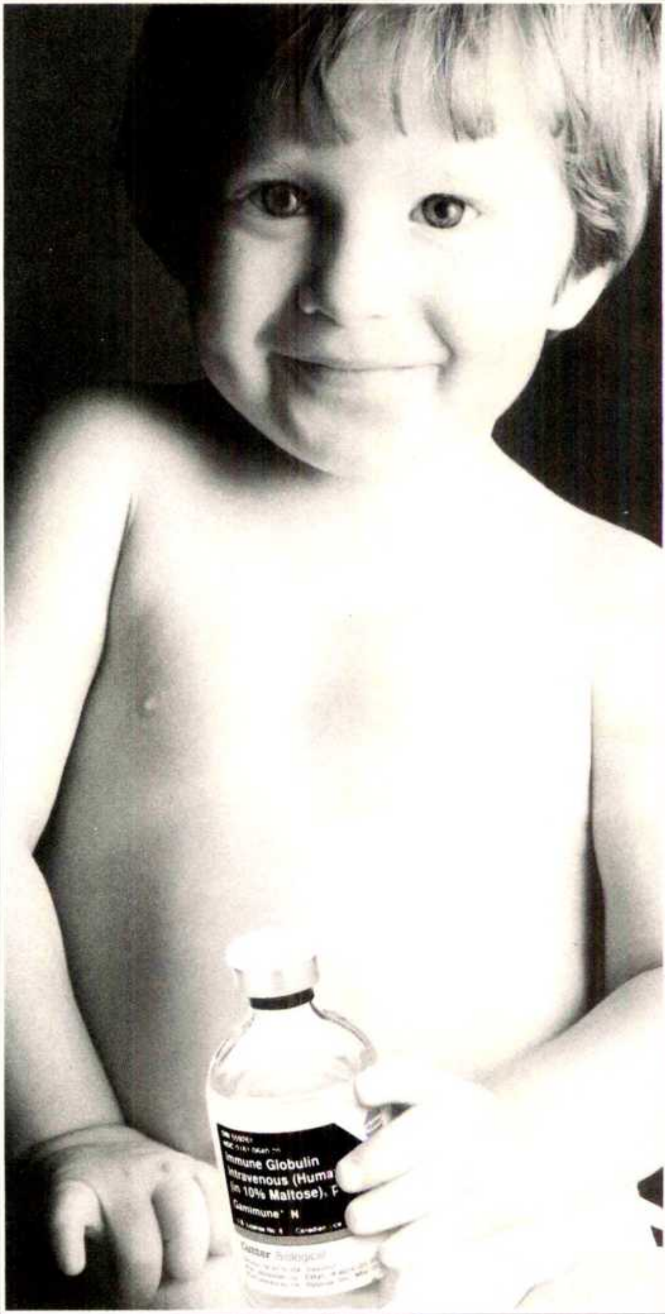

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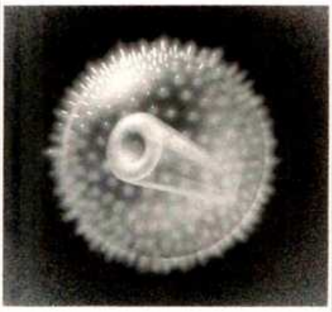
[‡]The Cohn-Oncley process used to produce GAMIMUNE® N has been challenged *in vitro* with a virus spike of 1 x 10^5 of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, GAMIMUNE® N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 reduction of HTLV-III.[3] In a separate study of intravenous immune globulins conducted by the Food and Drug Administration, an *in vitro* challenge at each of the six Cohn-Oncley fractionation steps using HTLV-III demonstrated a cumulative reduction of greater than 1 x 10^{15} infectious units.[4]



Artist's interpretation of HTLV-III virus.

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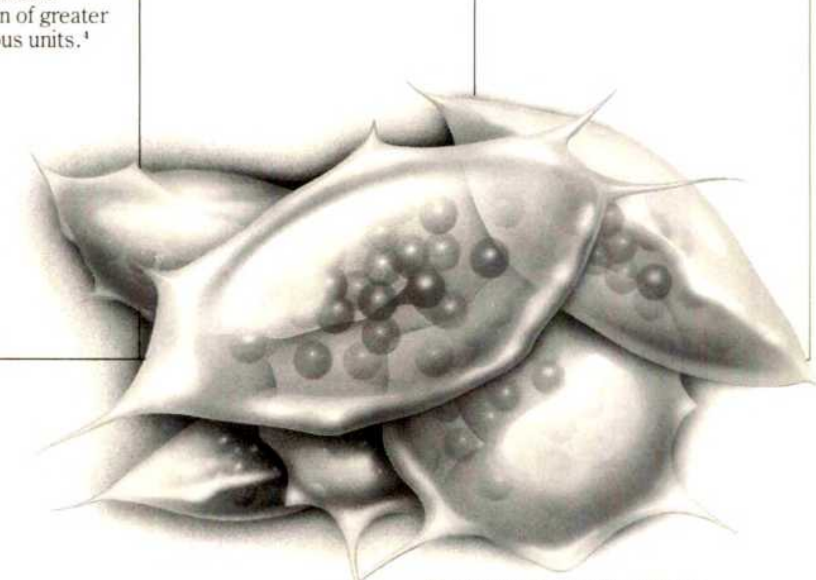

Artist's interpretation of blood platelets.

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Please see following page for brief summary of prescribing information.

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[*] It is not possible to predict which patients with ITP will respond to therapy.

[†] In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: *Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.*

Idiopathic Thrombocytopenic Purpura (ITP): *Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.*

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: *Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.*

Drug Interactions: *If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.*

Pregnancy Category C: *Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.*

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: *The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.*

Idiopathic Thrombocytopenic Purpura: *An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.*

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be ***gradually*** *increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.*

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. *GAMIMUNE® N Product License Application.* **2.** *Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. Rev Infect Dis 1986;8(Jul–Aug): S382–S390.* **3.** *Mitra G, Wong MF, Mosen MM, et al: Elimination of infectious retrovirus during preparation of immunoglobulins. Submitted for publication in Transfusion.* **4.** *Wells MA, Wittek AE, Epstein JS, et al: Inactivation and partition of human T-cell lymphotropic virus, type III, during ethanol fractionation of plasma. Transfusion 1986; 26(2): 210–213.*

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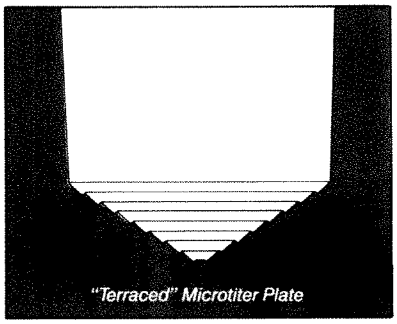
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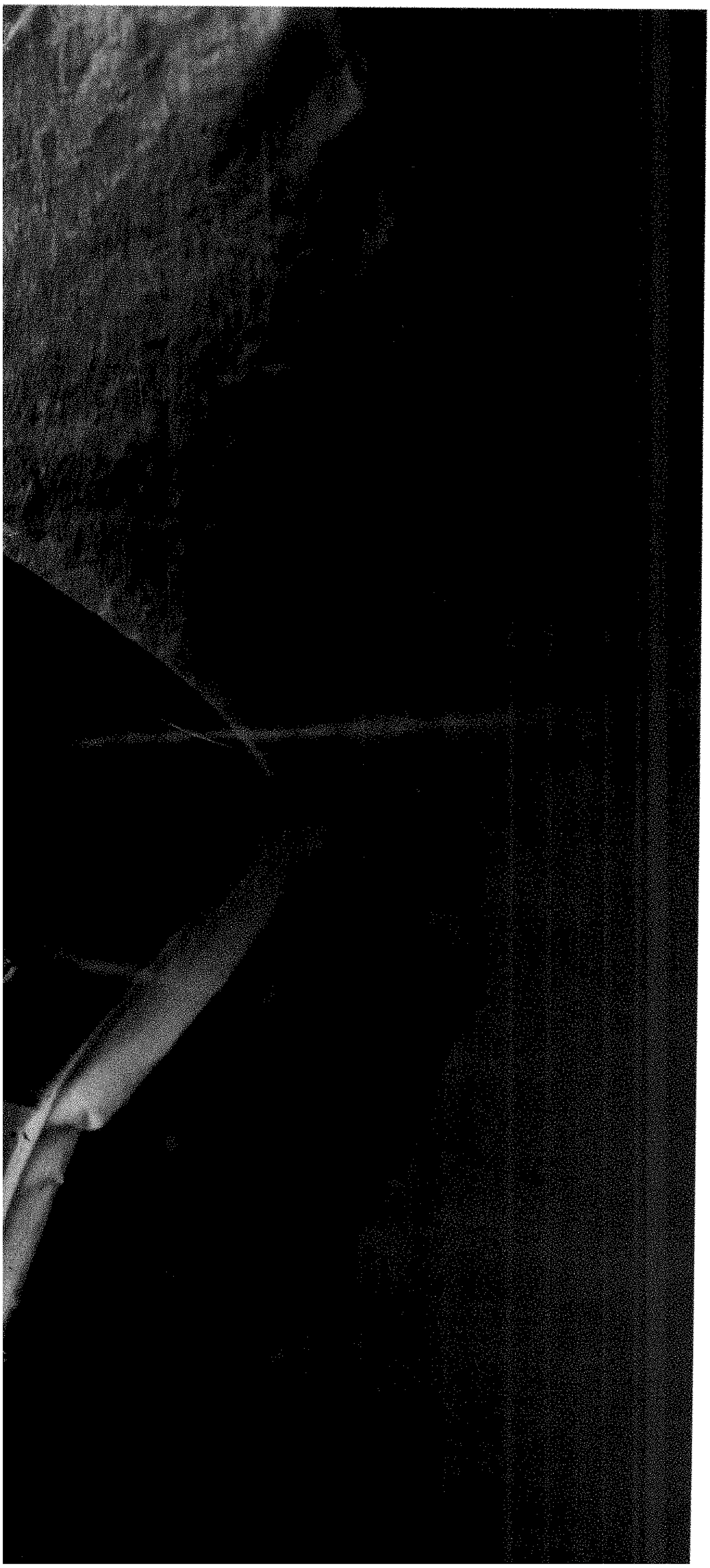

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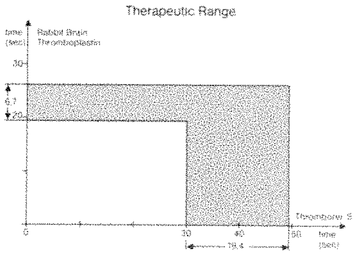

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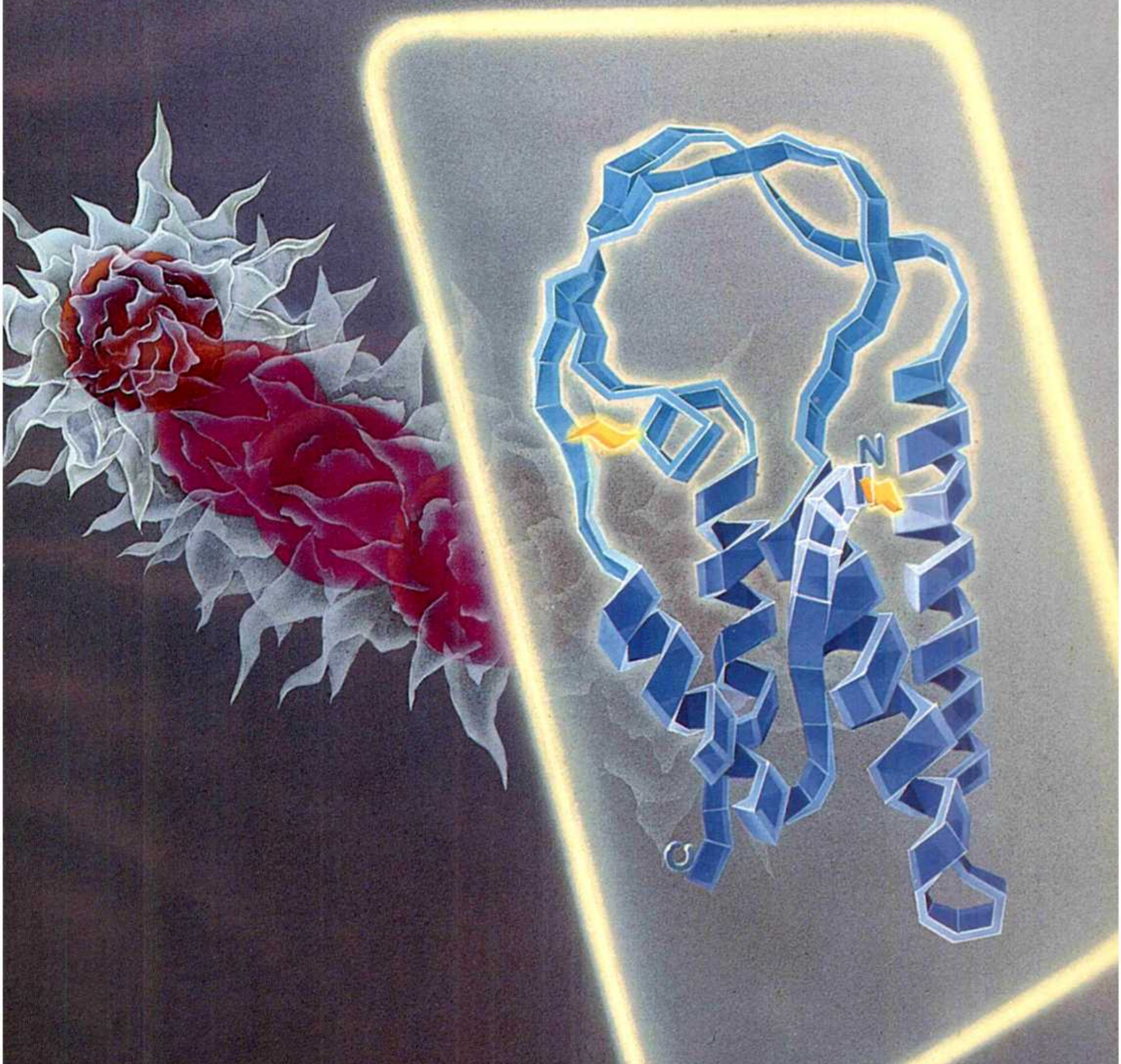

NEW INTRON® A

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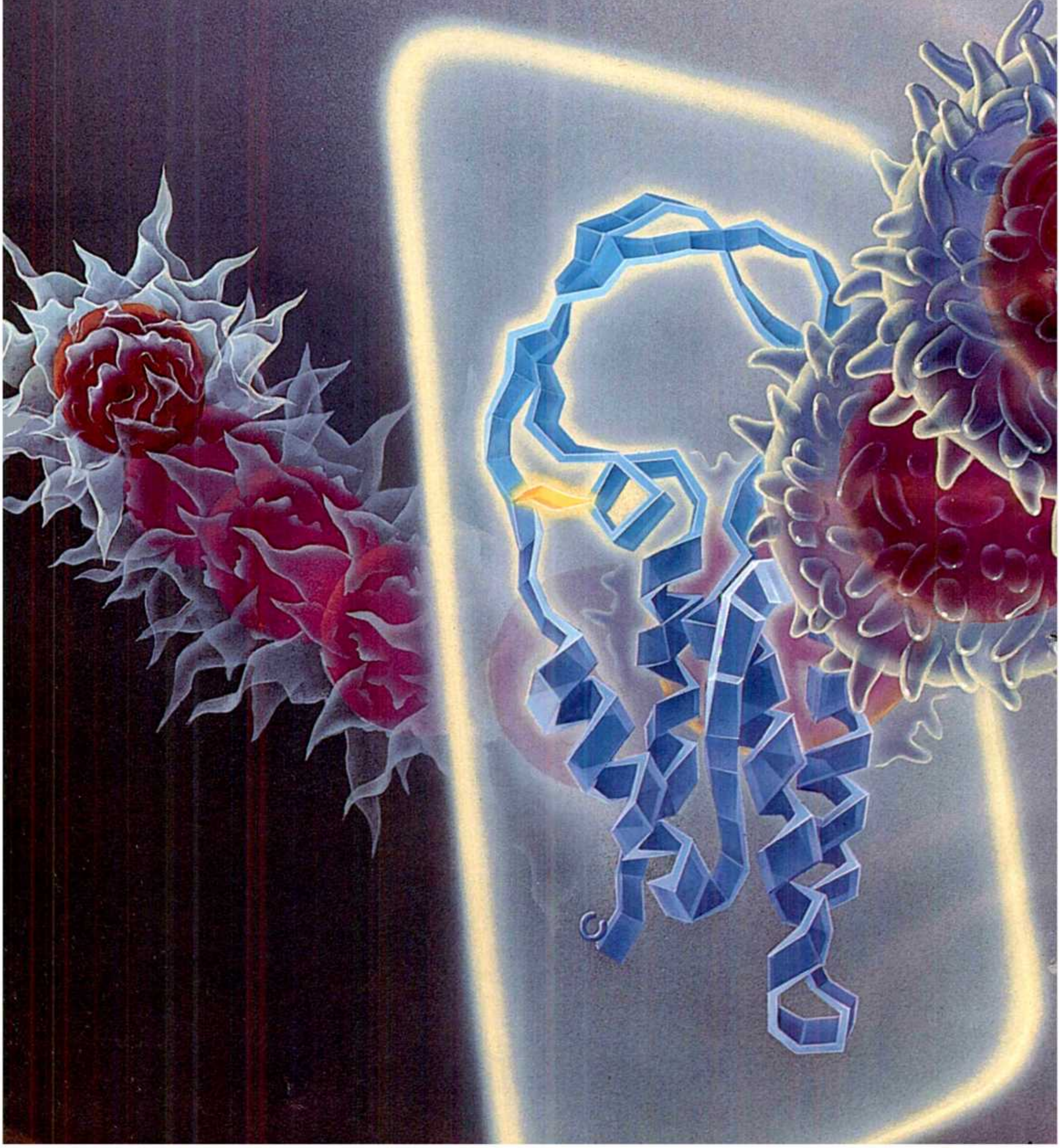
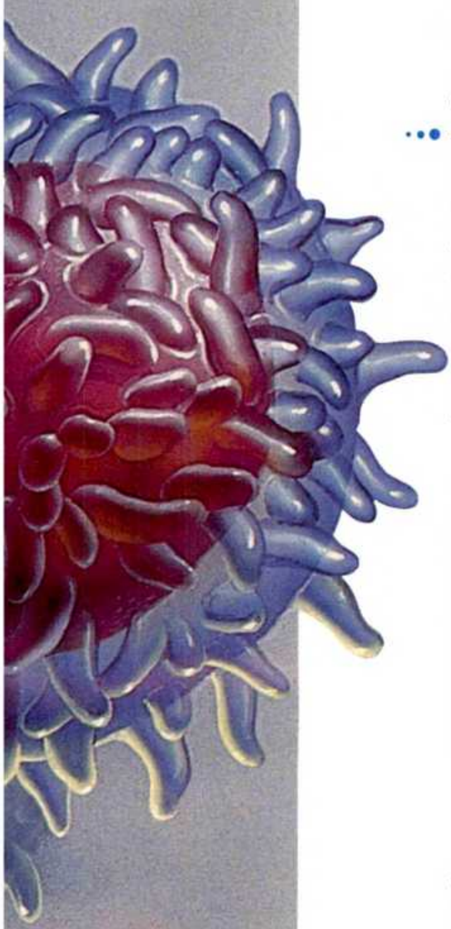
CHANGING THE COURSE OF HAIRY CELL LEUKEMIA



EFFICACY WELL ESTABLISHED

The efficacy of INTRON A was established in a 26-center clinical trial of 145 patients with progressive hairy cell leukemia, a B-cell malignancy.[1,2,3] INTRON A achieved a 74% objective response, measured as normalization of granulocyte, hemoglobin and platelet values and elimination of transfusion dependency.[1]



92% PROBABILITY OF SURVIVAL

In a separate analysis, a 24-month comparison was conducted between the INTRON A study population and historical controls.* After three months of therapy, patients receiving INTRON A reached and maintained a plateau of 92% probability of survival. The probability of survival among historical controls, however, declined steadily to 40% at 24 months.[1]

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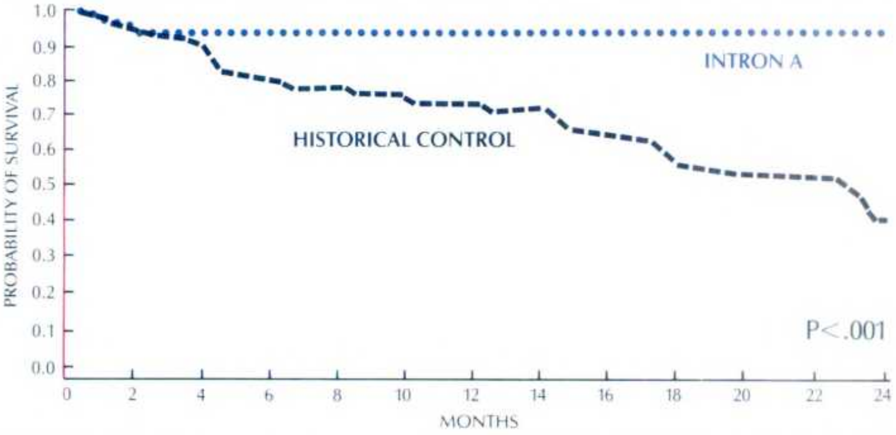

*Seventy-one historical control patients were treated with current standard therapies, 85% with chemotherapy (mainly chlorambucil) and 15% with supportive care.

DRAMATIC CLINICAL IMPROVEMENTS

More than 80% of the study population achieved normal levels of platelets by three months, of hemoglobin by six months and of granulocytes by twelve months. Marked decreases in the incidence of life-threatening infections and transfusion dependency preceded normalization of hematologic values.[1]

THERAPY WELL TOLERATED

Treatment-related side effects were generally mild to moderate and rapidly reversible upon discontinuation of therapy. The predominant complaint was a flu-like syndrome, which usually diminished with continued therapy. Mild-to-moderate skin rashes occurred intermittently in 40% of the study population. A low incidence of mild-to-moderate GI disorders was also reported.[1]

For a brief summary of prescribing information, please see the following page.

NEW INTRON® A

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INTRON® A Interferon alfa-2b recombinant for Injection

BRIEF SUMMARY

INDICATIONS AND USAGE INTRON A for Injection is indicated for the treatment of patients 18 years of age or older with hairy cell leukemia. Studies have shown that INTRON A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and non-splenectomized patients.

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically during treatment to determine whether response to treatment has occurred. If a patient does not respond within 6 months, treatment should be discontinued. If a response to treatment does occur, treatment usually should be continued until no further improvement is observed and these laboratory parameters have been stable for about 3 months (see **DOSAGE AND ADMINISTRATION**). It is not known whether continued treatment after that time point is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS INTRON A is contraindicated in patients with a history of hypersensitivity to interferon alfa or any component of the injection.

WARNINGS Moderate to severe adverse experiences may require modification of the patient's dosage regimen, or in some cases, termination of therapy with INTRON A.

Because of the fever and other "flu-like" symptoms associated with INTRON A administration, it should be used cautiously in patients with debilitating medical conditions, such as those with a history of cardiovascular disease (e.g., unstable angina, uncontrolled congestive heart failure), pulmonary disease (e.g., chronic obstructive pulmonary disease), or diabetes mellitus prone to ketoacidosis. Caution should also be observed in patients with coagulation disorders (e.g., thrombophlebitis, pulmonary embolism) or severe myelosuppression.

Patients with platelet counts of less than 50,000/mm^3 should not be administered INTRON A intramuscularly, but instead by subcutaneous administration.

Cardiovascular adverse experiences which include significant hypotension, arrhythmia, or tachycardia of 150 beats per minute or greater, were observed in approximately 3% of the patients studied who had various malignancies and were treated at doses higher than those for hairy cell leukemia. The incidence of these complications in patients with preexisting heart disease is unknown. Hypotension may occur during administration, or up to two days post therapy, and may require supportive therapy including fluid replacement to maintain intravascular volume. Supraventricular arrhythmias occurred rarely and appeared to be correlated with preexisting conditions, and prior therapy with cardiotoxic agents. These adverse experiences were controlled by modifying the dose or discontinuing treatment, but may require specific additional therapy.

Those patients with a recent history of myocardial infarction and/or previous or current arrhythmic disorder, who require INTRON A therapy, should be closely monitored (see **Laboratory Tests**).

Central nervous system effects manifested by depression, confusion and other alterations of mental status were observed in about 2% of hairy cell leukemia patients treated with INTRON A. The overall incidence in a larger patient population with other malignancies treated with higher doses of INTRON A was 10%. More significant obtundation and coma have been observed in some patients, usually elderly, treated at higher doses for other malignant diseases. These effects are usually rapidly reversible. In a few severe episodes, full resolution of symptoms has taken up to three weeks. Patients should be closely monitored until resolution of these effects. Discontinuation of INTRON A Interferon alfa-2b, recombinant for Injection therapy may be required. Narcotics, hypnotics, or sedatives may be used concurrently with caution.

Acute serious hypersensitivity reactions (e.g., urticaria, angioedema, bronchoconstriction, anaphylaxis, etc.) have not been observed in patients receiving INTRON A; however, if such an acute reaction develops, the drug should be discontinued immediately and appropriate medical therapy instituted. Transient cutaneous rashes have occurred in some patients following injection, but have not necessitated treatment interruption.

Laboratory abnormalities which occurred in hairy cell leukemia patients included elevated SGOT and SGPT, which occurred in 4% and 13% of patients, respectively. The overall incidences of laboratory abnormalities in a larger patient population with other malignancies, and treated at higher doses were somewhat higher. These included elevated liver function tests (SGOT, SGPT in 10% of patients) and reductions in granulocyte (20% of patients) and platelet counts (18% of patients) (see **Laboratory Tests**). These abnormalities are usually mild to moderate and transient. Severe abnormalities of these laboratory parameters are usually rapidly reversible upon cessation or reduction of INTRON A therapy.

PRECAUTIONS Information for Patients Patients being treated with INTRON A should be directed in its appropriate use, informed of benefits and risks associated with treatment and referred to the Patient Information Sheet.

Patients should be cautioned not to change brands of Interferon without medical consultation as a change in dosage may result.

It is advised that patients be well hydrated especially during the initial stages of treatment.

Laboratory Tests In addition to those tests normally required for monitoring patients with hairy cell leukemia, the following laboratory tests are recommended for all patients on INTRON A therapy, prior to beginning treatment and then periodically thereafter:

- Standard hematologic tests — including complete blood counts and differential as well as platelet counts.
- Blood chemistries — electrolytes and liver function tests.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer, should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis, Impairment of Fertility Studies with INTRON A have not been performed to determine carcinogenicity, or the effect on fertility. Interferon may impair fertility. Mutagenicity studies with INTRON A revealed no adverse effects.

Studies in mice, rats, and monkeys injected with INTRON A for up to one month have revealed no evidence of toxicity. However, due to the known species-specificity of interferon, the effects in animals are unlikely to be predictive of those in man.

Pregnancy Category C Animal reproduction studies have not been conducted with INTRON A. It is also not known whether INTRON A can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. INTRON A should be given to a pregnant woman only if clearly needed. Another interferon alfa preparation has been shown to have abortifacient effects in *Macaca mulatta* (rhesus monkeys) when given at 20 to 500 times the human dose. Therefore, INTRON A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. In studies of interferon use in non-human primates, abnormalities of the menstrual cycle have been observed.

INTRON A Interferon alfa-2b, recombinant for Injection should be used with caution in fertile men and women.

Nursing Mothers It is not known whether this drug is excreted in human milk. However, studies in mice have shown that mouse interferons are excreted into the milk. Because of the potential for serious adverse reactions from INTRON A in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use Safety and effectiveness have not been established in patients below the age of 18 years.

ADVERSE REACTIONS Adverse reactions to INTRON A are dose-related. In studies of patients with malignancies other than hairy cell leukemia, hematologic and hepatic toxicities were more common in patients receiving individual injections greater than 10 million IU, than in patients receiving less than this amount. Hematologic, hepatic, cardiovascular and neurologic toxicities were more common in patients receiving more than 30 million IU per injection than in patients receiving less than that amount.

The following adverse reactions were reported to be possibly or probably related to INTRON A therapy during clinical trials. The most frequently reported adverse reactions were flu-like symptoms, primarily fever, fatigue, and chills which occurred in almost all patients treated. Other organ systems in which reactions occurred in more than 5% of the patients are:

General — Taste alteration, anorexia and weight decrease occurred uncommonly.

Cardiovascular — Mild hypotension was frequently recorded although it was rarely symptomatic. Hypertension also occurred less commonly. Tachycardias occurred uncommonly usually in association with high fevers.

Gastrointestinal System — Nausea was common, although vomiting occurred rarely. Mild diarrhea was reported less commonly.

Hematologic — Mild thrombocytopenia and transient granulocytopenia occurred commonly.

Musculo-Skeletal System — Myalgia and arthralgia occurred commonly, usually in association with other flu-like symptoms.

Nervous System — Headache, somnolence, confusion, and dizziness occurred uncommonly. Ataxia and paresthesia were rare.

Psychiatric — Anxiety, depression and nervousness occurred uncommonly.

Skin and Appendages — Mild pruritus occurred uncommonly. Mild alopecia was uncommon. Various transient, mild skin rashes were seen frequently in patients with hairy cell leukemia.

Other Adverse Reactions — The following adverse reactions were reported with an incidence of 5% or less: leg cramps, constipation, insomnia, herpetic eruptions, non-herpetic cold sores, urticaria, hot flashes, supraventricular arrhythmias, epistaxis, stomatitis, paralytic ileus, dehydration, coagulation disorder (elevated PT and PTT), abnormal vision, tremor, emotional lability, chest pain, pharyngitis and syncope.

Adverse reactions reported rarely (less than 1%) were dyspepsia, purpura, dyspnea, sneezing, oculomotor paralysis, nasal congestion, flatulence, increased saliva, hyperglycemia, and ulcerative stomatitis.

Laboratory Value Changes — Those patient laboratory values which were normal to moderately abnormal (WHO grades 0–2) at baseline that worsened to either severe or life-threatening abnormalities (WHO grades 3 or 4) during some phase of treatment included: WBC, platelets, granulocytes, SGOT, creatinine, SGPT, LDH, and alkaline phosphatase.

DOSAGE AND ADMINISTRATION The recommended dosage of INTRON A for Injection for the treatment of hairy cell leukemia is 2 million IU/m^2 administered intramuscularly (see **WARNINGS**) or subcutaneously 3 times a week. Higher doses are not recommended. The normalization of one or more hematologic variables usually begins within 2 months of initiation of therapy. Improvement in all three hematologic variables may require 6 months or more of therapy.

This dosage regimen should be maintained unless the disease progresses rapidly, or severe intolerance is manifested. If severe adverse reactions develop, the dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. If persistent or recurrent intolerance develops following adequate dosage adjustment, or disease progresses, treatment with INTRON A Interferon alfa-2b, recombinant for Injection should be discontinued. The minimum effective dose of INTRON A has not been established.

At the discretion of the physician, the patient may self-administer the dose. The medication may be administered at bedtime.

HOW SUPPLIED INTRON A Interferon alfa-2b, recombinant for Injection is available in 3-, 5-, 10- and 25-million IU vials, each packaged with its own diluent.

Store INTRON A for Injection, both before and after reconstitution, between 2° and 8°C (36° and 46°F).

JBS-13762805

References: **1.** Data on file, Schering Corporation. **2.** Jacobs AD, Champlin RE, Golde DW: Recombinant α-2-interferon for hairy cell leukemia. *Blood* 1985; 65:1017-1020. **3.** Golomb HM, Jacobs A, Fefer A, et al: Interferon treatment of HCL. *J Clin Oncol*. To be published. **4.** Herberman RB, Ortaldo JR, Rubinstein M, et al: Augmentation of natural and antibody-dependent cell-mediated cytotoxicity by pure human leukocyte interferon. *J Clin Oncol* 1981; 1:149-153. **5.** Schultz RM: Macrophage activation by interferons. *Lymphokine Reports* 1980; 1:63-97. **6.** Herberman RB, Ortaldo JR, Mantovani A, et al: Effect of human recombinant interferon on cytotoxic activity of natural killer (NK) cells and monocytes. *Cell Immunol* 1982; 67:160-167. **7.** Tomida M, Yamamoto Y, Hozumi M: Stimulation by interferon of induction of differentiation of human promyelocytic leukemia cells. *Biochem Biophys Res Comm* 1982; 104(Jan 15):30-37. **8.** Samid D, Chang EH, Friedman RM: Biochemical correlates of phenotypic reversion in interferon-treated mouse cells transformed by a human oncogene. *Biochem Biophys Res Comm* 1984; 119(Feb 29):21-28. **9.** Brouty-Boyé D, Wybier-Franqui J, Calvo C, et al: Reversibility of the transformed and neoplastic phenotype. IV. Effects of long-term interferon treatment of C3H/10T½ cells transformed by methylcholanthrene and SV40 virus. *Int J Cancer* 1984; 34:107-112. **10.** Friedman RM: *Interferons: A Primer*. New York, Academic Press, 1981, pp 3-7. **11.** Jonak GJ, Knight E Jr: Selective reduction of c-*myc* mRNA in Daudi cells by human β interferon. *Proc Natl Acad Sci* 1984; 81:1747-1750. **12.** Soslau G, Bogucki AR, Gillespie D, et al: Phosphoproteins altered by antiproliferative doses of human interferon-β in a human bladder carcinoma cell line. *Biochem Biophys Res Comm* 1984; 119:941-948. **13.** Balkwill F, Taylor-Papadimitriou J: Interferon affects both G_1 and $S + G_2$ in cells stimulated from quiescence to growth. *Nature* 1978; 274:798-800. **14.** Salmon SE, Durie BGM, Young L, et al: Effects of cloned human leukocyte interferons in the human tumor stem cell assay. *J Clin Oncol* 1983; 1:217-225. **15.** Welander CE, Morgan TM, Homesley HD, et al: Combined recombinant human interferon alpha_2 and cytotoxic agents studied in a clonogenic assay. *Int J Cancer* 1985; 35:721-729.

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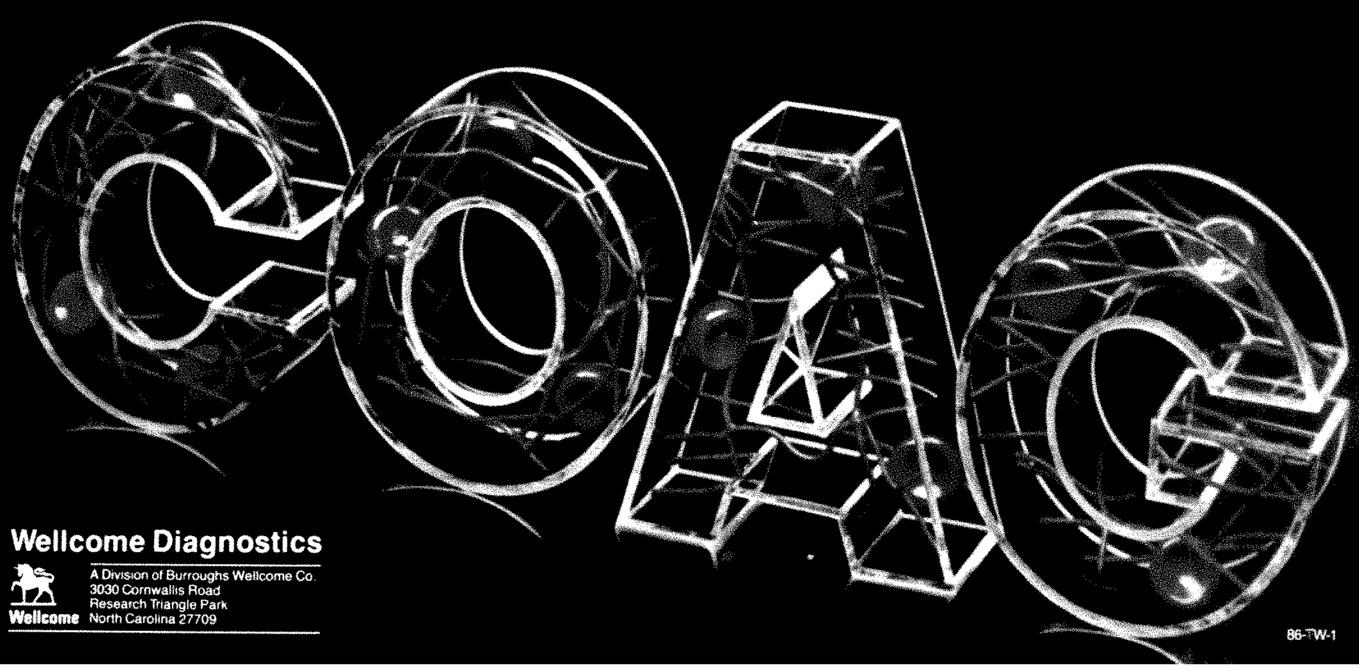

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GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

*The Cohn-Oncley process used to produce GAMIMUNE® N has been challenged *in vitro* with a virus spike of 1 x 10^5 of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, GAMIMUNE® N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 reduction of HTLV-III.[2] In a separate study of intravenous immune globulins conducted by the Food and Drug Administration, an *in vitro* challenge at each of the six Cohn-Oncley fractionation steps using HTLV-III demonstrated a cumulative reduction of greater than 1 x 10^{15} infectious units.[3]

Please see following page for brief summary of prescribing information.

GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days, there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. GAMIMUNE® N Package Insert. **2.** Mitra G, Wong MF, Mosen MM, et al: Elimination of infectious retrovirus during preparation of immunoglobulins. Submitted for publication in Transfusion. **3.** Wells MA, Wittek AE, Epstein JS, et al: Inactivation and partition of human T-cell lymphotropic virus, type III, during ethanol fractionation of plasma. Transfusion 1986; 26(2): 210–213.

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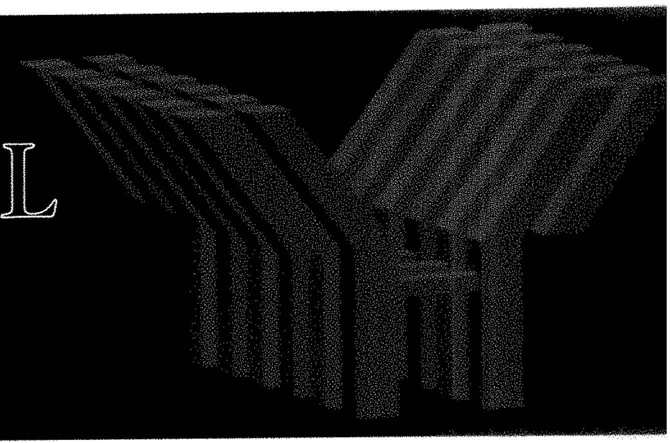

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press:

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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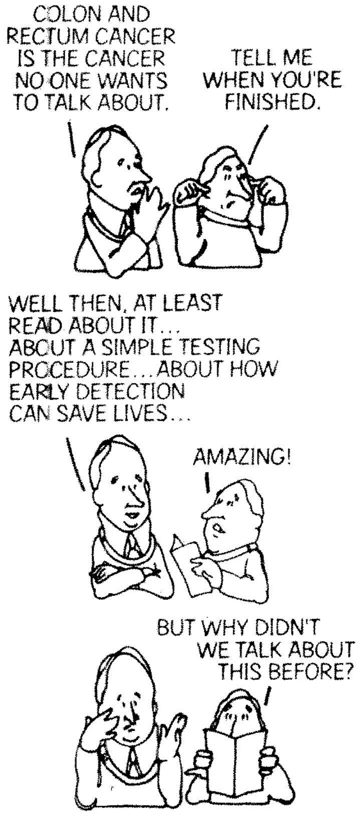
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Today, ours is the only IGIV *not contraindicated* in selective IgA deficiency.

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***Burks, A. W., Sampson, H. A., and Buckley, R. H. (1986):** *Anaphylactic Reactions after Gamma Globulin Administration in Patients with Hypogammaglobulinemia.* **New England Journal of Medicine** 314 (9): 560-564

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See the following page for full prescribing information.

DIRECTION INSERT

Immune Globulin Intravenous (Human)
GAMMAGARD®

DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® * is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

REFERENCES

1. Unpublished data in the files of Travenol Laboratories, Inc.
2. Waldmann TA, Storber W: Metabolism of immunoglobulins. *Prog Allergy 13*: 1-110, 1969
3. Morell A, Riesen W: Structure, function and catabolism of immunoglobulins in *Immunohemotherapy*. Nydegger UE (ed), London, Academic Press, 1981, pp 17-26
4. Stiehm ER: Standard and special human immune serum globulins as therapeutic agents. *Pediatrics* 63:301-319, 1979
5. Buckley RH: Immunoglobulin replacement therapy: indications and contraindications for use and variable IgG levels achieved in *Immunoglobulins: Characteristics and Use of Intravenous Preparations*. Alving BM, Finlayson JS (eds), Washington, DC, U.S. Department of Health and Human Services, 1979, pp3-8
6. Burks AW, Sampson HA, Buckley RH: Anaphylactic reactions following gammaglobulin administration in patients with hypogammaglobulinemia: detection of IgE antibodies to IgA. Submitted for publication.

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MACOP-B

Drug Doses and Treatment Schedule for MACOP-B†

Drug	Dose	Route	Timing
Methotrexate††	400 mg/m^2	IV	Weeks 2, 6, 10
Doxorubicin	50 mg/m^2	IV	Weeks 1, 3, 5, 7, 9, 11
Cyclophosphamide	350 mg/m^2	IV	Weeks 1, 3, 5, 7, 9, 11
Vincristine	1.4 mg/m^2	IV	Weeks 2, 4, 6, 8, 10, 12
Bleomycin	10 U/m^2	IV	Weeks 4, 8, 12
Prednisone	75 mg	Oral	Daily, dose tapered over the last 15 days
Co-trimoxazole	2 tablets	Oral	Twice daily throughout

† MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; IV = intravenous.

†† Methotrexate given as a 100 mg/m^2 intravenous bolus, then 300 mg/m^2 intravenously over 4 hours, followed 24 hours later by folinic acid, 15 mg orally, every 6 hours for 6 doses.

* Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med*. 102:596-602, 1985.

Blenoxane®
(sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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POORLY DIFFERENTIATED NEOPLASMS AND TUMORS OF UNKNOWN ORIGIN

Edited by

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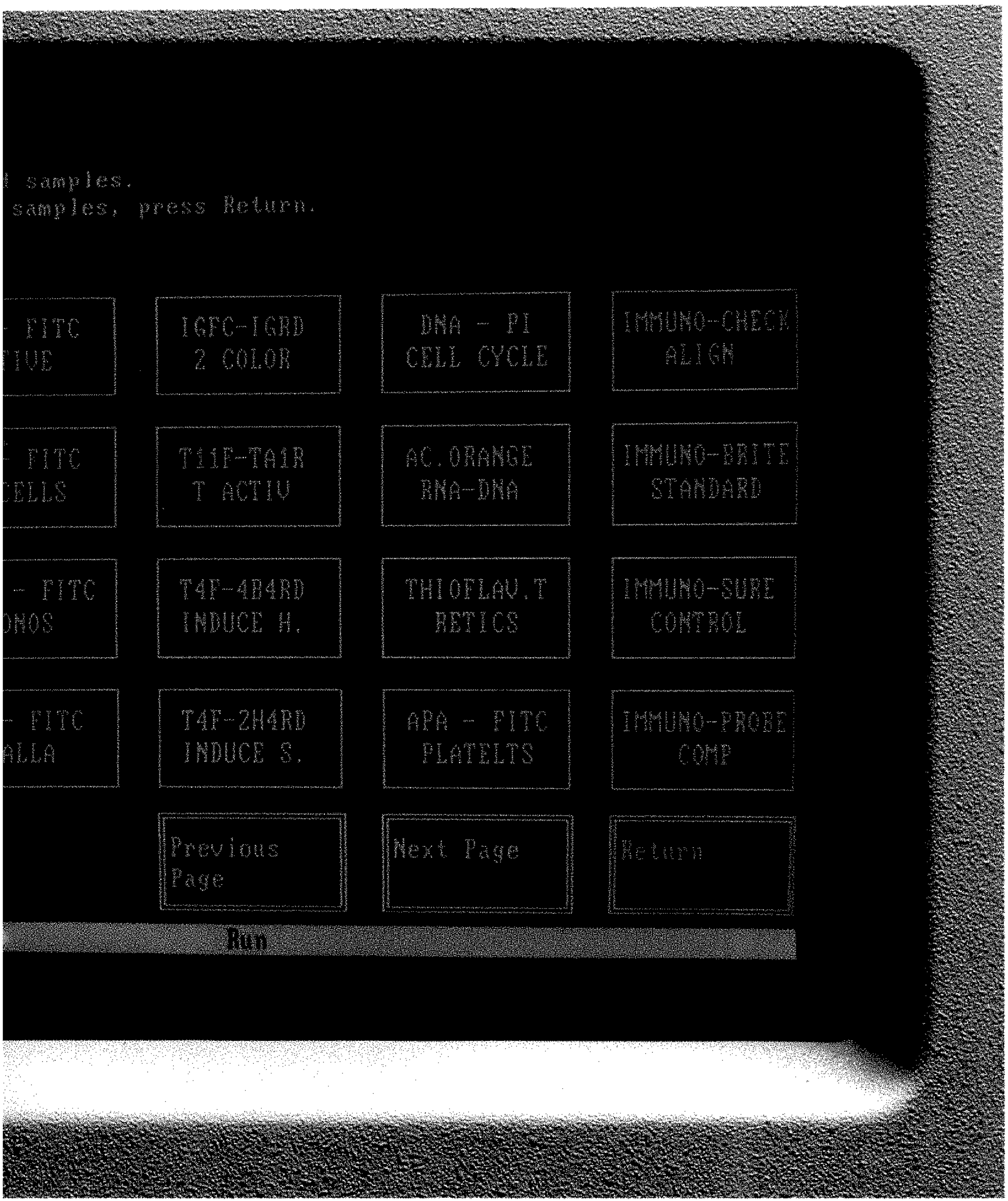

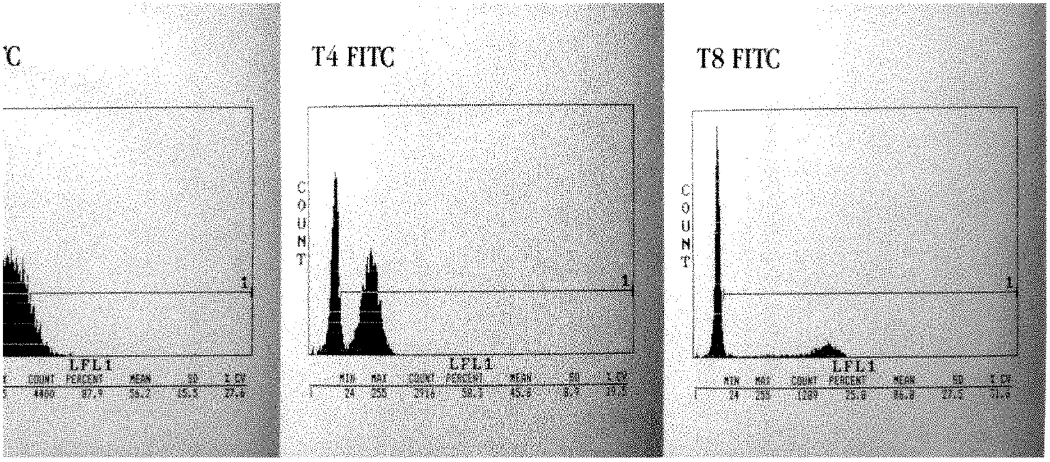

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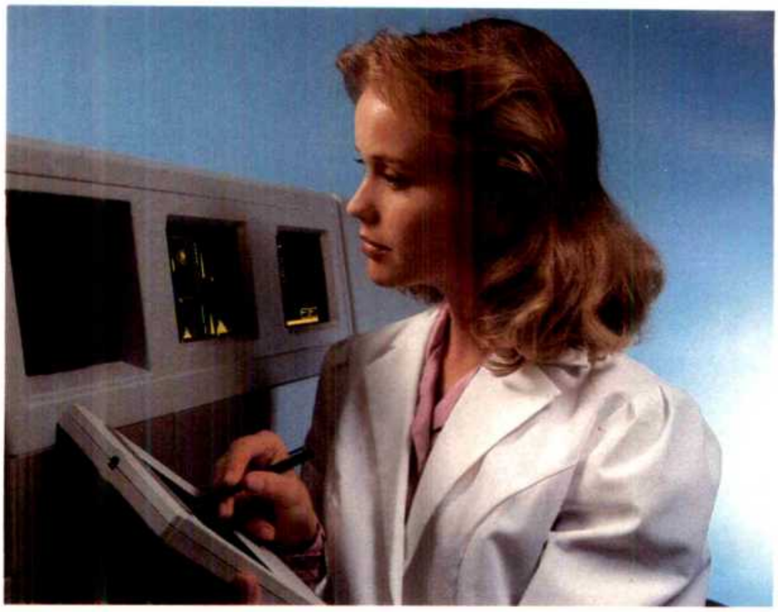

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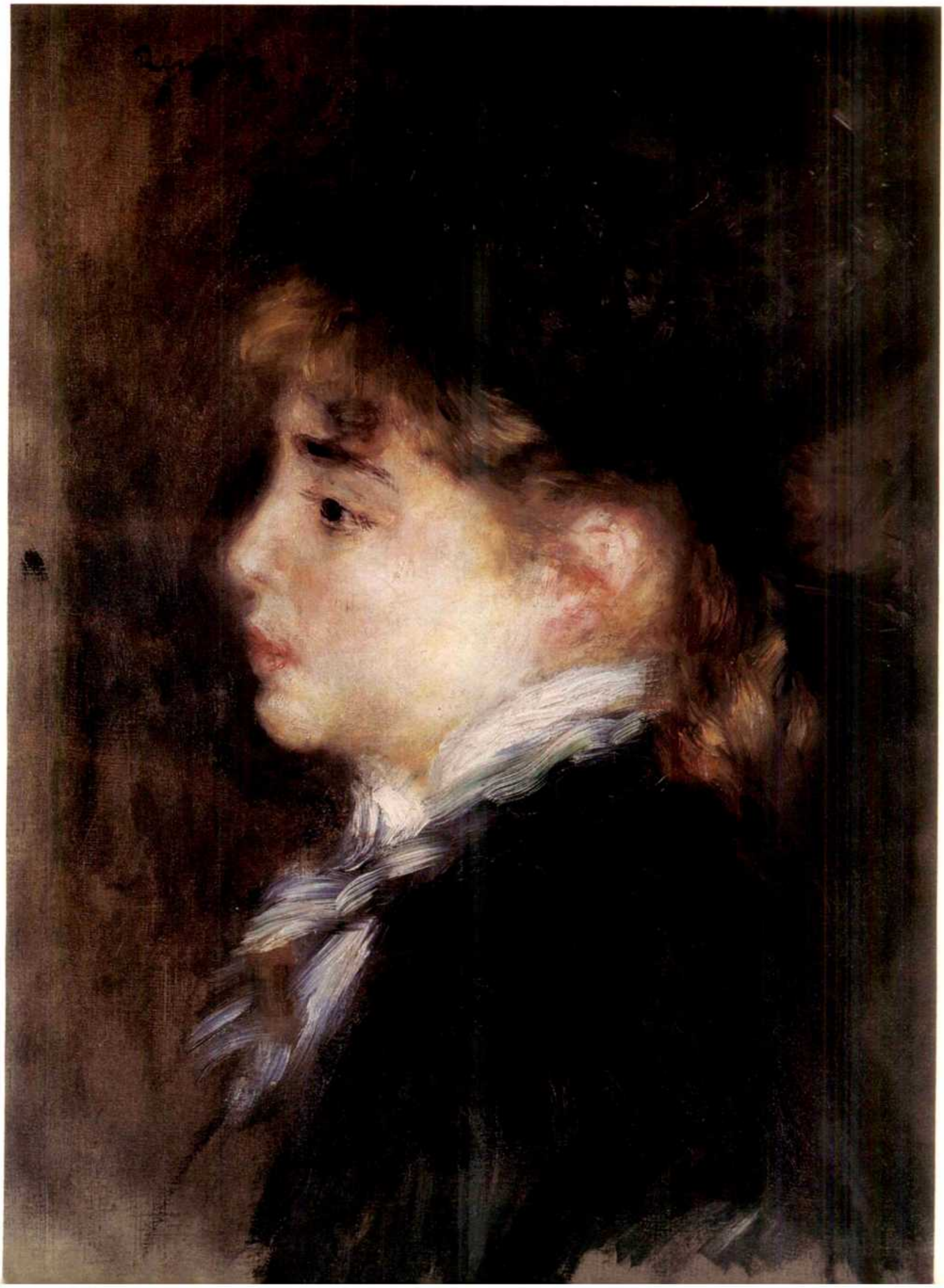

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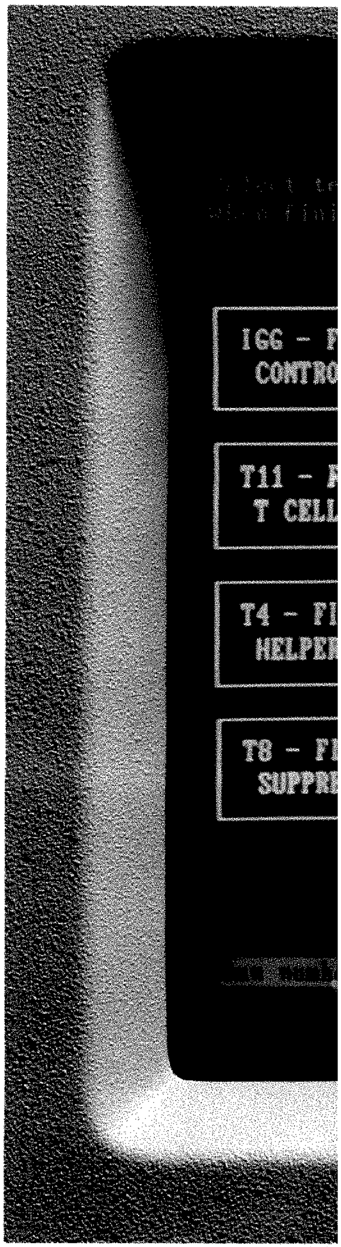

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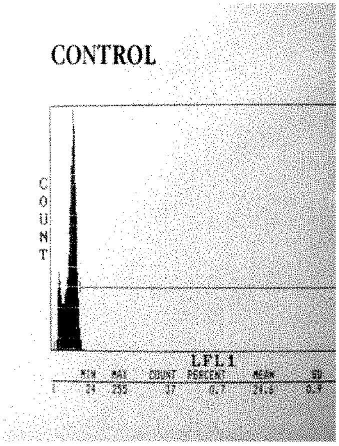

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PROGRESS IN HEMOSTASIS AND THROMBOSIS, VOLUME VIII

Edited by

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Professor of Medicine and Pathology
Division of Hematology
State University of New York at Stony Brook
Health Sciences Center
Stony Brook, New York

Progress in Hemostasis and Thrombosis, Volume 8, carries on the tradition established for the series under Dr. Theodore Spaet's editorship, of publishing definitive reviews on major topics of current interest in the broad fields of hemostasis and thrombosis.

For the first edition under his editorship, Dr. Barry S. Coller has selected a rich mixture of both basic and clinical topics written by authors with outstanding international reputations. Several chapters deal extensively with the new therapeutic modalities, including thrombolysis with tissue plasminogen activators, intravenous gamma-globulin, and DDAVP. Among the other topics covered are: the hereditary thrombophilias; Factor VIII; Protein Kinase C; Transcellular Metabolism of Eicosenoids; Porcine von Willebrand's Disease; and Heparin-like Molecules of the Endothelium.

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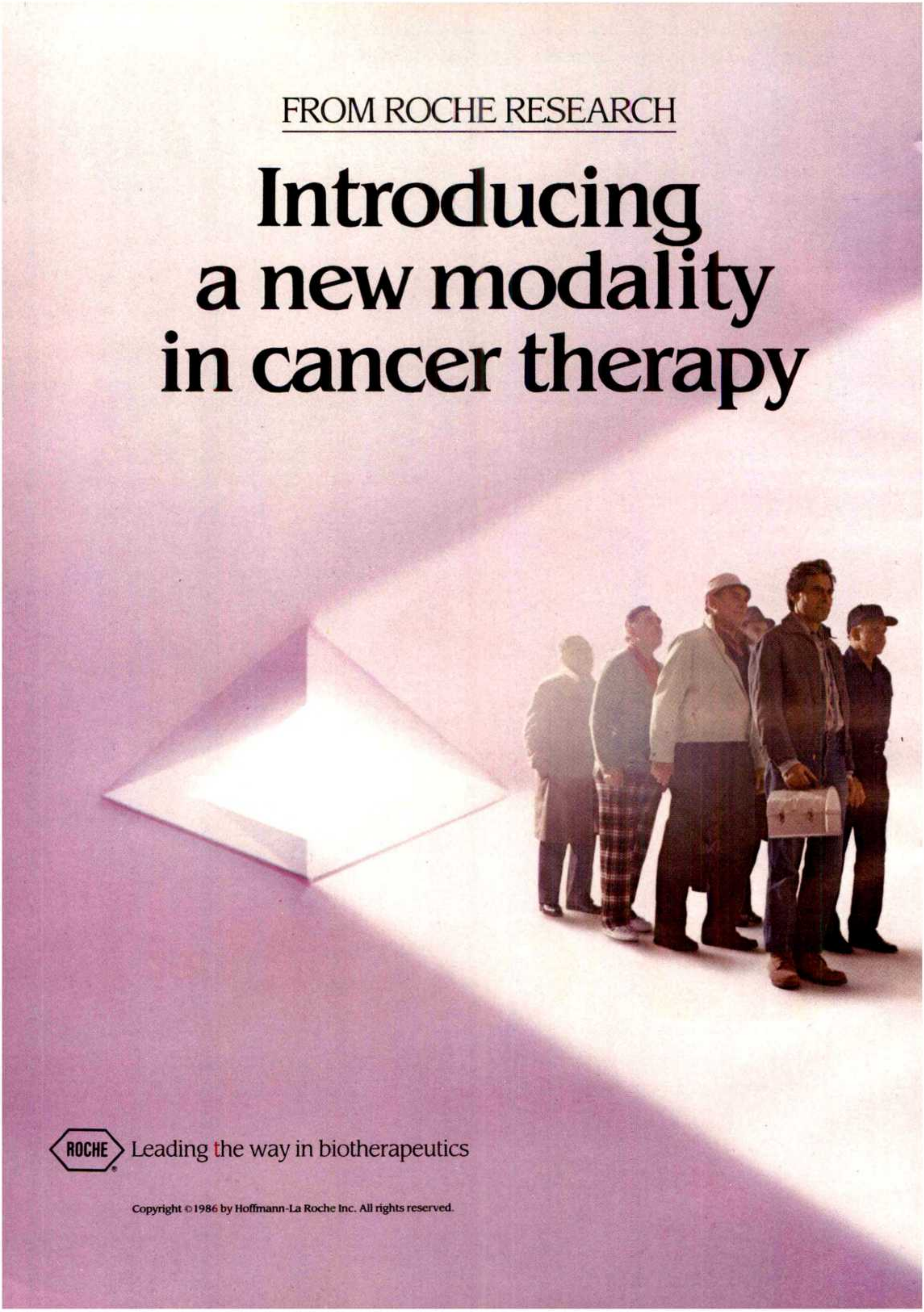
FROM ROCHE RESEARCH
Introducing a new modality in cancer therapy
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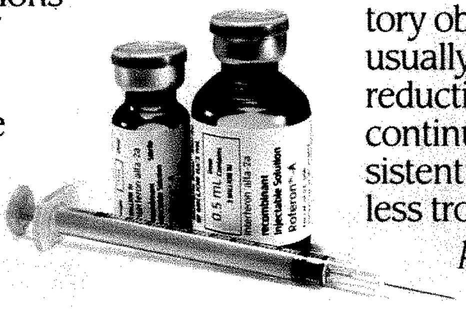
a breakthrough in hairy cell leukemia

- ☐ Effective in all stages of the disease.
- ☐ Statistically increased survival compared to a historical control group.
- ☐ Normalizes peripheral blood elements.
- ☐ Improves quality of life.
- ☐ Dramatically decreases the need for red blood cell and platelet transfusions.
- ☐ Virtually eliminates life-threatening opportunistic infections after several months of therapy.
- ☐ Ready-to-use injectable solution. No mixing or reconstitution necessary.
- ☐ SC or IM injection and fixed dosing permit administration without weight or body surface area calculation in home, office or institution.
- ☐ Caution patients not to change brands of Interferon without medical consultation, as a change in dosage may result.
- ☐ Common initial side effects, such as fever, chills, myalgia, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug. Persistent fatigue is found to be less troublesome with *p.m.* or *h.s.* administration. See following pages for details on all adverse reactions.



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NEW SC/IM

ROFERON®-A

Interferon alfa-2a, recombinant/Roche

a promise fulfilled

Before prescribing, please see complete product information on following pages.

NEW SC/IM

ROFERON®-A

BRAND OF
Interferon alfa-2a, recombinant/Roche

DESCRIPTION: Roferon*-A (Interferon alfa-2a, recombinant/Roche) is a sterile protein product for use by injection. Roferon-A is manufactured by recombinant DNA technology that employs a genetically engineered *E. coli* bacterium containing DNA that codes for the human protein. Interferon alfa-2a, recombinant/Roche is a highly purified protein containing 165 amino acids, and it has an approximate molecular weight of 19,000 daltons. The purification procedure includes affinity chromatography using a murine monoclonal antibody. Roferon-A is supplied as an injectable solution. The solution is colorless and contains Interferon alfa-2a, recombinant/Roche, phenol as a preservative, sodium chloride for isotonicity and human serum albumin as a stabilizer. Fermentation is carried out in a defined nutrient medium containing the antibiotic tetracycline hydrochloride, 5 mg/liter. However, the presence of the antibiotic is not detectable in the final product.

Each vial of Roferon-A contains 3 million or 18 million international units (IU) of Interferon alfa-2a, recombinant/Roche. The specific activity of Interferon alfa-2a, recombinant/Roche, is 2×10^8 IU/mg protein. The route of administration is subcutaneous or intramuscular.

CLINICAL PHARMACOLOGY: The mechanism by which Interferon alfa-2a recombinant/Roche, or any other interferon, exerts antitumor activity is not clearly understood. However, it is believed that direct antiproliferative action against tumor cells and modulation of the host immune response play important roles in the antitumor activity.

The biological activities of Interferon alfa-2a, recombinant/Roche are species-restricted, *i.e.*, they are expressed in a very limited number of species other than humans. As a consequence, preclinical evaluation of Interferon alfa-2a, recombinant/Roche has involved *in vitro* experiments with human cells and some *in vivo* experiments.[1] Using human cells in culture, Interferon alfa-2a, recombinant/Roche has been shown to have antiproliferative and immunomodulatory activities that are very similar to those of the mixture of interferon alfa subtypes produced by human leukocytes. *In vivo*, Interferon alfa-2a, recombinant/Roche has been shown to inhibit the growth of several human tumors growing in immunocompromised (nude) mice. Because of its species-restricted activity, it has not been possible to demonstrate antitumor activity in immunologically intact syngeneic tumor model systems, where effects on the host immune system would be observable. However, such antitumor activity has been repeatedly demonstrated with, for example, mouse interferon-alfa in transplantable mouse tumor systems. The clinical significance of these findings is unknown.

The metabolism of Interferon alfa-2a, recombinant/Roche is consistent with that of alfa interferons in general. Alfa interferons are totally filtered through the glomeruli and undergo rapid proteolytic degradation during tubular reabsorption, rendering a negligible reappearance of intact alfa interferon in the systemic circulation. Small amounts of radiolabeled Interferon alfa-2a, recombinant/Roche appear in the urine of isolated rat kidneys, suggesting near complete reabsorption of Interferon alfa-2a, recombinant/Roche catabolites. Liver metabolism and subsequent biliary excretion are considered minor pathways of elimination for alfa interferons.

The serum concentrations of Interferon alfa-2a, recombinant/Roche reflected a large intersubject variation in both healthy volunteers and patients with disseminated cancer.

In healthy people, Interferon alfa-2a, recombinant/Roche exhibited an elimination half-life of 3.7 to 8.5 hours (mean 5.1 hours), volume of distribution at steady-state of 0.223 to 0.748 L/kg (mean 0.400 L/kg) and a total body clearance of 2.14 to 3.62 mL/min/kg (mean 2.79 mL/min/kg) after a 36 million IU (2.2×10^8 pg) intravenous infusion. After intramuscular and subcutaneous administrations of 36 million IU, peak serum concentrations ranged from 1500 to 2580 pg/mL (mean 2020 pg/mL) at a mean time to peak of 3.8 hours and from 1250 to 2320 pg/mL (mean 1730 pg/mL) at a mean time to peak of 7.3 hours, respectively. The apparent fraction of the dose absorbed after intramuscular injection was greater than 80%.

The pharmacokinetics of Interferon alfa-2a, recombinant/Roche after single intramuscular doses to patients with disseminated cancer were similar to those found in healthy volunteers. Dose proportional increases in serum concentrations were observed after single doses up to 198 million IU. There were no changes in the distribution or elimination of Interferon alfa-2a, recombinant/Roche during twice daily (0.5 to 36 million IU), once daily (1 to 54 million IU), or three times weekly (1 to 136 million IU) dosing regimens up to 28 days of dosing. Multiple intramuscular doses of Interferon alfa-2a, recombinant/Roche resulted in an accumulation of 2 to 4 times the single dose serum concentrations. Pharmacokinetic information in patients with hairy cell leukemia is presently unknown.

The acute parenteral toxicity of Interferon alfa-2a, recombinant/Roche has been studied in mice, rats, rabbits and ferrets at doses up to 30 million IU/kg intravenously, and 500 million IU/kg intramuscularly. No treatment-related mortality was noted in any species given Interferon alfa-2a, recombinant/Roche by any of the routes of administration.

Effects on Hairy Cell Leukemia: During the first one to two months of treatment of patients with hairy cell leukemia, significant depression of hematopoiesis was likely to occur. Subsequently, there was improvement in circulating blood cell counts.

Of the 75 patients who were evaluable for efficacy following at least 16 weeks of therapy, 46 (61%) achieved complete or partial response. Twenty-one patients (28%) had a minor remission, eight (11%) remained stable, and none had worsening of disease. All patients who achieved either a complete or partial response had complete or partial normalization of all peripheral blood elements including hemoglobin level, white blood cell, neutrophil, monocyte and platelet counts with a concomitant decrease in peripheral blood and bone marrow hairy cells. Responding patients also exhibited a marked reduction in red blood cell and platelet transfusion requirements, a decrease in infectious episodes and improvement in performance status. The probability of survival for two years in patients receiving Roferon-A (94%) was statistically increased compared to a historical control group (75%).

ROFERON*-A (Interferon alfa-2a, recombinant/Roche)

INDICATIONS AND USAGE: Roferon-A is indicated for use in the treatment of hairy cell leukemia in people 18 years of age or older. Studies have shown that Roferon-A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and nonsplenectomized patients.[2,3]

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically (*e.g.*, monthly) during treatment to determine whether response to treatment has occurred. If a patient does not respond within six months, treatment should be discontinued. If a response to treatment does occur, treatment should be continued until no further improvement is observed and these laboratory parameters have been stable for about three months. It is not known whether continued treatment after that time is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS: Roferon-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Roferon-A should be administered under the guidance of a qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of the therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function.

Because of the possibility of severe or even fatal adverse reactions, patients should be informed not only of the benefits of therapy but also of the risks involved.

Roferon-A should be administered with caution to patients with cardiac disease or with any history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (*i.e.*, fever, chills) frequently associated with Roferon-A administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred in patients receiving Roferon-A.

Caution should be exercised when administering Roferon-A to patients with myelosuppression.

Central nervous system adverse reactions have been reported in a number of patients. These reactions included decreased mental status, exaggerated central nervous system function, and dizziness. More severe obtundation and coma have been rarely observed. Most of these abnormalities were mild and reversible within a few days to three weeks upon dose reduction or discontinuation of Roferon-A therapy. Careful periodic neuropsychiatric monitoring of all patients is recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* In all instances where the use of Roferon-A is considered for chemotherapy, the physician must evaluate the need and usefulness of the drug against the risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken according to the clinical judgment of the physician. Reinstitution of Roferon-A therapy should be carried out with caution and with adequate consideration of the further need for the drug and alertness as to possible recurrence of toxicity.

The minimum effective dose of Roferon-A for treatment of hairy cell leukemia has not been established.

Information for Patient: Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. Patients should be informed regarding the potential benefits and risks attendant to the use of Roferon-A. If home use is determined to be desirable by the physician, instructions on appropriate use should be given, including review of the contents of the enclosed Patient Information Sheet. Patients should be well hydrated, especially during the initial stages of treatment.

Laboratory Tests: Periodic complete blood counts and liver function tests should be performed during the course of Roferon-A treatment. They should be performed prior to therapy and at appropriate periods during therapy. Since responses of hairy cell leukemia are not generally observed for one to three months after initiation of treatment, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase of treatment.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility:

Carcinogenesis: Roferon-A has not been tested for its carcinogenic potential.

Mutagenesis: A. Internal studies—Ames tests using six different tester strains, with and without metabolic activation, were performed with Roferon-A up to a concentration of 1920 µg/plate. There was no evidence of mutagenicity.

Human lymphocyte cultures were treated *in vitro* with Roferon-A at noncytotoxic concentrations. No increase in the incidence of chromosomal damage was noted.

B. Published studies—There are no published studies on the mutagenic potential of Roferon-A. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported.

A chromosomal defect following the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder has been reported.

In contrast, other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon.

It has also been shown that human leukocyte interferon protects primary chick embryo fibroblasts from chromosomal aberrations produced by gamma rays.

Impairment of Fertility: Roferon-A has been studied for its effect on fertility in Macaca mulatta (rhesus monkeys). Nonpregnant rhesus females treated with Roferon-A at doses of 5 and 25 million IU/kg/day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. These monkeys returned to a normal menstrual rhythm following discontinuation of treatment.

Drug Interactions: Interactions between Roferon-A and other drugs have not been fully evaluated.

PREGNANCY: *Teratogenic Effects:* Pregnancy Category C. Roferon-A has been shown to demonstrate a statistically significant increase in abortifacient activity in rhesus monkeys when given at approximately 20 to 500 times the human dose. A study in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation) has failed to demonstrate teratogenic activity for Roferon-A. There are no adequate and well-controlled studies in pregnant women.
Nonteratogenic Effects: Dose-related abortifacient activity was observed in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation). A late-fetal period study (days 79 to 100 of gestation) is in progress and as yet there have been no reports of any increased rate of abortion.
Usage in Pregnancy: Safe use in human pregnancy has not been established. Therefore, Roferon-A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Information from primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during the therapy period.

Male fertility and teratologic evaluations have yielded no significant adverse effects to date.
Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Roferon-A, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.
Pediatric Use: Safety and effectiveness in children under 18 years of age have not been established.
ADVERSE REACTIONS: The following data on adverse reactions are based on the subcutaneous or intramuscular administration of Roferon-A as a single agent for the treatment of 63 patients with hairy cell leukemia during investigational trials in the United States.
Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%) and headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), dryness or inflammation of the oropharynx (16%), dry skin or pruritus (13%) and weight loss (14%) were observed with moderate frequency. Less commonly, diaphoresis (8%), paresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of herpes labialis (8%), transient impotence (6%) and arthralgias (5%) were also observed. Rarely (<3%), central nervous system effects including decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias and palpitations, were reported.
Adverse experiences which occurred rarely and may have been related to underlying disease, included epistaxis, bleeding gums, ecchymosis and petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis and inflammation at the site of injection, were also rarely observed.
Roferon-A has also been evaluated for the treatment of many other types of cancer under investigational trials in the United States. These studies generally utilized higher doses (12 to 50 million IU/m^2). All of the previously described adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in patients who received higher doses of Roferon-A. Some additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%) and edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: *Gastrointestinal*—abdominal fullness, hypermotility and hepatitis; *Central Nervous System*—gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia and loss of libido; *Peripheral Nervous System*—muscle contractions; *Cardiovascular*—congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon and hot flashes; *Pulmonary*—bronchospasm and tachypnea; *Miscellaneous*—excessive salivation and flushing of skin.
Abnormal Laboratory Test Values: The percentage of patients with hairy cell leukemia or with other types of malignancies who experienced a significant abnormal laboratory test value at least once during their treatment is shown in the following table:

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary[*] (n = 1019)
Hematologic		
Leukopenia	59%	69%
Neutropenia	39%	58%
Thrombocytopenia	42%	42%
Decreased Hemoglobin	36%	6.3%
Decreased Hematocrit	43%	12.5%
Mean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
Hepatic		
SGOT	47%	78%
Alkaline Phosphatase	18%	48%
LDH	12%	47%
Bilirubin	1.6%	31%
Renal/Urinary		
BUN	2%	10%
Serum Creatinine	3%	10%
Uric Acid[†]	6%	15%
Proteinuria[†]	10%	25%
Other Chemistry Tests		
Hypocalcemia	10%	51%
Elevated FBS[‡]	33%	39%
Elevated Serum Phosphorus	2%	17%

[*] Patients with hairy cell leukemia are included in this overall summary.

[†] The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.

[‡] Random glucose determination.

Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.[4]
DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended.
Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.
If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.
DIRECTIONS FOR USE: The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count <50,000) or who are at risk for bleeding.
Injectable Solution:

3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative.

18 million IU Roferon-A per vial (for multiple-dose use)—Each 1 mL contains 6 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche.

The injectable solution should be stored in the refrigerator at 36° to 46°F (2° to 8°C). Do *not* freeze. Do not shake.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.
HOW SUPPLIED: Sterile Roferon-A is supplied as single and multiple-dose vials:
Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01).
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REFERENCES:

1. Trown PW *et al: Cancer 57*(Suppl.):1648-1656, 1986.
2. Foon KA *et al: Blood 64*(Suppl. 1):164a, 1984.
3. Quesada JR *et al: Cancer 57*(Suppl.):1678-1680, 1986.
4. Jones GJ, Itri LM: *Cancer 57*(Suppl.):1709-1715, 1986.

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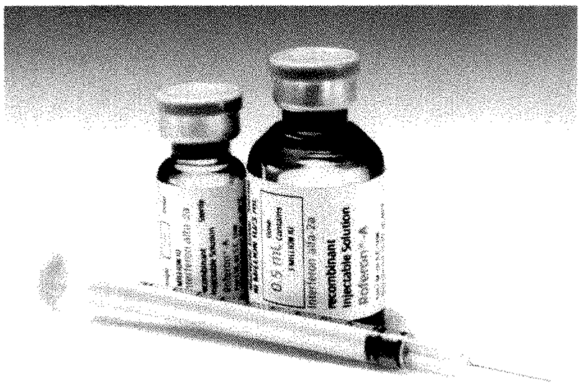

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ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists (See Warnings) (b) In the treatment of the megaloblastic anemias due to sprue, nutritional deficiency pregnancy, and infancy when oral therapy is not feasible

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12} A hematologic remission may occur while neurologic manifestations remain progressive

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid

DOSAGE: Megaloblastic anemia: No more than or up to 1 mg daily There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg, additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg

For the treatment of overdosage of folic acid antagonists To be given in amounts equal to the weight of the antagonist given

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LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

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INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS)

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12} A hematologic remission may occur while neurologic manifestations remain progressive

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes

PRECAUTIONS: *General* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney

Drug Interactions Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children

Pregnancy Teratogenic Effects Pregnancy Category C Animal reproduction studies have not been conducted with leucovorin It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity Leucovorin should be given to a pregnant woman only if clearly needed

Nursing Mothers It is not known whether this drug is excreted in human milk Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother

Pediatric Use (See Drug Interactions)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase Leucovorin rescue must begin within 24 hours of antifolate administration A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below $5 \times 10^{-8}M$ [7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators [9,10,11]

REFERENCES:
1 Nixon PF and Bertino JR Effective Absorption and Utilization of Oral Formyltetrahydrofolate in Man *N Engl J Med 286*(4) 175-179, Jan 1972
2 Ratanasthien K, Blair JA, Leeming RJ, Cooke WT and Melikian V Folates in Human Serum *J Clin Pathol 27* 875-879, 1974
3 Mehta BM, Gisolfi AL, Hutchison DJ Nirenberg A, Kellick MG and Rosen G Serum Distribution of Citrovorum Factor and 5-methyltetrahydrofolate Following Oral and I M Administration of Calcium Leucovorin in Normal Adults *Cancer Treat Rep 62*(3) 345-350, Mar 1978
4 Data on file, Clinical Research, Lederle Laboratories, Pearl River NY 10965
5 Whitehead VM, Pratt R, Viallet A and Cooper BA Intestinal Conversion of Folinic Acid to 5-methyltetrahydrofolate in Man *Br J Haematol 22* 63-72, 1972
6 Herbert V Aseptic Addition for *Lactobacillus casei* Assay of Folate Activity in Human Serum *J Clin Path 19* 12-16, 1966
7 Bleyer WA The Clinical Pharmacology of Methotrexate *Cancer 41*(1) 36-51 1978
8 Frei E Blum RH, Pitman SW, *et al* High Dose Methotrexate with Leucovorin Rescue Rationale and Spectrum of Antitumor Activity *Am J Med 68* 370-376, 1980
9 Golde DW, Bersch N, Quan SG Trimethoprim and Sulphamethoxazole Inhibition of Haematopoiesis *in Vitro Br J Haematol, 40*(3) 363-367, 1978
10 Steinberg SE, Campbell CL, Rabinovitch PS, *et al* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells *Blood*, 55(3) 501-504, 1980
11 Mahmoud AAF and Warren KS Algorithms in the Diagnosis and Management of Exotic Disease XX Toxoplasmosis *J Infect Dis 135*(3) 493-496, 1977

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REVIEW

The Liabilities of Iron Deficiency

By James D. Cook and Sean R. Lynch

Iron has been recognized as a potent hematinic since the inception of hematology as a clinical discipline. Even ancient civilizations believed in the beneficial effects of medicinal iron.[1] Nevertheless, the precise functional liabilities of iron lack remain the subject of continuing debate. The consequences of iron deficiency, particularly from a socioeconomic standpoint, are especially important in light of its high global prevalence. A recent review of the literature indicates that ~30% of the estimated world population of nearly 4.5 billion are anemic, and at least half of these, 500 to 600 million people, are believed to have iron deficiency anemia.[2]

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FROM BOTH a clinical and a laboratory standpoint, iron deficiency is usually recognized by the anemia that accompanies its later stages. It is therefore hardly surprising that the liabilities of iron deficiency are often viewed only in terms of a decrease in circulating hemoglobin. Although one of the most important functions of iron is its role in oxygen transport and storage, iron participates in a wide variety of biochemical processes, including mitochondrial electron transport, catacholamine metabolism, and DNA synthesis. Not surprisingly, a broad spectrum of biochemical abnormalities resulting from iron deficiency has been described.[3-6] A large number of enzymes are known to contain iron or require it as a co-factor, including cytochrome oxidase, succinate dehydrogenase, aconitase, catalase, myeloperoxidase, cytochrome C reductase, ribonucleotide reductase, tyrosine hydroxylase, and xanthine oxidase. Although it has been difficult to relate the tissue concentrations of these enzymes to the functional consequences of iron deficiency, a depletion in tissue iron may be as important as a reduction in circulating hemoglobin.[5,7]

The following review focuses on the nonhematological liabilities of iron deficiency in humans, with emphasis on clinical and socioeconomic implications. The effects of iron deficiency on gastrointestinal structure and function are not discussed in detail because these have been discussed extensively in the classical literature and recent reviews.[3,4,6] The tissues of the gastrointestinal tract are particularly susceptible to the effects of severe chronic iron depletion, which may explain the clinical manifestations of glossitis, stomatitis, esophageal webs, chronic gastritis, and malabsorption. Of these now uncommon manifestations, atrophic gastritis is the most important because a reduction in gastric acidity perpetuates iron deficiency by impairing the assimilation of dietary iron. The key liabilities of tissue iron deficiency relate to abnormalities in host defense, work performance, and neurological function.

IMMUNITY AND INFECTION

There is much conflicting information about the relationship between iron status and infection.[8-10] It is commonly stated that iron deficiency increases the susceptibility to common infections by impairing certain host defense mechanisms such as cell-mediated immunity and phagocytosis. On the other hand, there is recent evidence that vigorous iron therapy may sometimes predispose to certain life-threatening infections. The following discussion is presented from a clinical perspective, although there are few if any well-controlled trials.

The earliest report suggesting that iron administration may reduce the frequency of common infections during infancy was published by MacKay.[11] In a subsequent report, a sharply lower incidence of respiratory infections was observed in infants from a low socioeconomic population who were given an iron-containing formula for periods ranging up to 9 months.[12] Later studies in otherwise healthy children with mild iron deficiency failed to demonstrate beneficial effects of iron.[13] In one recent study conducted in adults in Indonesia, a threefold reduction in the prevalence of enteritis and influenza-type illnesses was observed when workers on a rubber plantation were given 100 mg of ferrous sulfate daily for several weeks.[14] Similar trials were recently conducted in Egypt and Indonesia, but the results have not yet been made available.

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In certain infections, the role of mild iron deficiency is more evident. Iron deficiency is recognized as an important predisposing factor in chronic mucocutaneous candidiasis. Higgs and Wells[15] reported that 23 of 31 patients with this disorder showed evidence of mild iron deficiency that was confirmed in most cases by bone marrow examination. This striking association may be explained in part by the impairment in epithelial function and structure that is known to occur in persons with mild iron deficiency and may also account for the recently described association between iron deficiency and recurrent Herpes simplex infection. A significantly higher prevalence of iron deficiency as measured by transferrin saturation was observed among medical students with Herpes infection as compared with a group of healthy controls.[16]

The beneficial effect of iron therapy in reducing the incidence of mild infections apparently results from the correction of a defect in immune function. Whereas humoral immunity is normal in patients with iron lack, a defect in cell-mediated immunity has been demonstrated repeatedly. One of the earliest such observations was made by Joynson and co-workers,[17] who described an impairment in lymphocyte transformation and migration inhibition factor production on stimulation with Candida antigen and purified protein derivative in 12 subjects with iron deficiency anemia. Both the proportion and the absolute number of circulating T cells are commonly reduced in patients with iron deficiency anemia.[16] Lymphocyte proliferation and response to the mitogens phytohemagglutinin (PHA) and concanavalin A are impaired even in nonanemic patients with latent iron deficiency, and a significant correlation has been established between the lymphocyte stimulation index and transferrin saturation.[16,18,19] In a recent study in ten iron-deficient children aged 12 to 30 months, the mean stimulation indices for Candida antigen increased from 6.8% to 17.9% and for tetanus from 19.5% to 31.7% following iron therapy.[20] These various studies provide ample evidence for a significant if not profound defect in T cell immunity.

A defect in neutrophil function may also predispose patients with mild iron deficiency to repeated infections. Chandra[21] demonstrated that intracellular bacterial killing by polymorphonuclear leukocytes in 12 children with iron deficiency anemia was significantly impaired. The reduction of nitroblue tetrazolium was similarly reduced, although opsonic activity of the plasma and phagocytosis were normal. The defect in neutrophil function that occurs in persons with iron deficiency has been attributed to an impairment in myeloperoxidase activity.[22]

On the other hand, there is evidence that rapid alleviation of iron deficiency may promote certain bacterial and parasitic infections. It is well known that iron is an essential nutrient for microbial growth and that the multiplication of many pathogens is sharply curtailed in human tissue where they must compete with the iron-binding proteins transferrin and lactoferrin. The strong bacteriostatic and bacteriocidal qualities of serum can be readily overcome by saturating these iron-binding proteins with iron.[23] Moreover, the ability of many pathogens to acquire iron from their host is an important determinant of their virulence and the nature of the infection produced.[24] Several animal studies have indicated that pretreatment of the host with iron increases the range and severity of many bacterial infections, but the evidence that these observations have clinical relevance is still limited

In at least one clinical situation, heavy iron administration is associated with the development of serious infection. During the early 1970s, newborn Polynesian infants were given prophylactic intramuscular iron dextran in an effort to reduce the incidence of iron deficiency anemia During the 5-year period of this program, the incidence of neonatal sepsis in the infants given iron was 11 in 1,000 total births as compared with 0.6 in 1,000 in European infants, a dramatic 20-fold difference.[23] When this program of routine parenteral iron administration was stopped, the incidence of gram-negative neonatal sepsis fell from 17 to 2.7 in 1,000 births. Sepsis resulting from prophylactic iron was almost invariably due to *Escherichia coli* and occurred in >80% of cases between day 4 and day 10 following iron administration.[25] Several factors may have accounted for the pronounced increase in neonatal sepsis. For example, infection may have resulted solely from administration of a deep intramuscular injection in the newborn, or reticuloendothelial cell blockade may have been produced by the iron dextran complex.

Nevertheless, the sudden influx of iron into the circulation and tissues in a nonphysiological form cannot be excluded as the primary predisposing cause. Iron dextran remains in the circulation for several days following intramuscular injection and provides a source of iron not otherwise available to microorganisms. In addition, the iron released from the iron dextran complex reduces the level of unsaturated circulating transferrin and could thereby impair the bacteriostatic properties of the plasma against *E coli*.[26] Whatever the cause, this report indicates that parenteral iron should not be given to newborn infants prophylactically. At the same time, there is no evidence that parenteral iron is hazardous to healthy persons with a competent immune system. Hundreds of women have been given parenteral iron during pregnancy with no recorded evidence of an increase in bacterial sepsis.

The hypothesis that iron deficiency may serve as a useful defense mechanism by withholding iron from bacteria, fungi, or protozoa has gained some tenuous support from recent studies in which iron was supplied to severely malnourished populations.[10] One commonly cited report is that of Masawe and co-workers[27] in which 100 anemic patients consecutively admitted to an adult medical ward in East Africa were studied for the frequency of infection. Approximately two-thirds of them had absent iron stores, whereas the remainder with stainable marrow iron had megaloblastic, hemolytic, or refractory anemia. It was noted that only 7% of the iron-deficient patients had bacterial infections, whereas between 64% and 83% with stainable iron had various bacterial infections. Although it was suggested that the iron-deficient state protects against the development of bacterial infections, these observations could also be explained by the more common occurrence of infections in anemic patients with normal or increased levels of body iron, such as those with sickle cell anemia or bone marrow failure. For example, hemolysis may promote bacterial growth by increasing iron supply in the form of heme compounds.[8] The only proper control subjects in a study of this kind would be patients with normal hemoglobin levels.

These same workers reported that 16 of 18 patients with malaria, proven by the identification of *Plasmodium falciparum,* had iron deficiency anemia and noted that malarial attacks occurred commonly in these patients following the institution of iron therapy. Evidence shows that iron therapy may predispose to the development of malaria in other population settings. Following treatment of iron deficiency in Somali nomads, a recrudescence of malaria was observed in 13 of 71 persons as compared with only 1 of 76 in a control group.[28] This phenomenon may be explained by an increased production of reticulocytes following iron therapy, with a resulting increase in malarial parasitemia. If the susceptibility to malaria is a consequence of reticulocytosis following iron therapy, a strong argument exists for the eradication of nutritional iron deficiency through programs of food iron fortification, thereby reducing the need to treat severely iron-deficient persons with medicinal iron.

It should be emphasized that these studies provide no evidence of a risk in treating iron deficiency in otherwise healthy populations or in persons who are not at risk for malarial infection Further carefully controlled epidemiologic studies are needed to define the risk of alleviating iron deficiency anemia in severely malnourished populations. Because iron deficiency anemia in the tropics leads to severe morbidity and is frequently fatal, a noted authority in tropical medicine and hematology has concluded that severely anemic patients should be treated with iron while being protected against malaria and be treated for bacterial infections as they arise.[29]

WORK PERFORMANCE

A significant limitation in certain types of physical activity has emerged as one of the most important consequences of chronic iron deficiency. This is certainly true from a socioeconomic if not a clinical standpoint. Interest in this facet of iron deficiency arises from studies in chronically anemic agricultural laborers engaged in physically demanding tasks in the lowlands of Guatemala.[30] When a large group of men were tested for their ability to perform near-maximal exercise of brief duration (Harvard Step Test), a striking correlation was demonstrated between mean hemoglobin concentration and this aerobic activity. The key finding was not that the severely anemic person had a limitation in exercise performance, but that this was also related to differences in hemoglobin levels within the normal range. When iron and folic acid were supplied to the anemic men, a progressive improvement in exercise performance was observed, attaining a maximum after 2 months of treatment. The difference was highly significant when the men were compared with a control group, although a slight improvement was observed in control subjects as well, due to a training effect. This study provided the first solid evidence that even mild degrees of iron deficiency can impair performance in occupations requiring a heavy energy expenditure.

Similar findings have been observed in other field settings and with other types of work activity. Physical work capacity was studied in 75 female subjects employed on a tea plantation in Sri Lanka.[31] Approximately two-thirds of them were anemic, with hemoglobin concentrations as low as 6 g/100 mL. Work performance was evaluated in a laboratory setting using a multistage treadmill test in which the percentage of grade was progressively increased at 3-minute intervals until a maximum possible work time of 18 minutes was attained. Performance improved at higher hemoglobin concentrations, both in total time on the treadmill and the percentage of persons reaching the highest workload. In addition, there was a striking correlation between mean hemoglobin concentration and the percentage of increase in mean heart rate when compared with values observed in subjects with a normal hemoglobin concentration ($r = .97$). As in the Guatemala studies, even mild anemia (hemoglobin concentration between 11 and 11.9 g/100 mL) was associated with a 20% decrease in work tolerance. The postexercise lactate level was also sharply higher in anemic women and correlated closely with the degree of anemia. The authors concluded that because of the dramatic impairment in work capacity and an inferred reduction in work productivity, a strong case could be made for the correction and prevention of iron deficiency on the basis of economic as well as health considerations.

These observations were translated into concrete economical terms by studies in latex tappers employed on an Indonesian rubber plantation.[14] Three hundred two male subjects aged 16–40 years were evaluated; 45% were anemic as judged by a hemoglobin level <13 g/100 mL. Hookworm infection was documented as the cause of the iron deficiency anemia in most subjects. Work output could be measured precisely in this study because each man was paid daily on the basis of the weight of latex he collected. Hemoglobin concentrations over the range of 10 to 16 g/100 mL correlated closely with income ($r = .56$). The daily latex output was 18.7% lower in anemic men; when they were given 100 mg elemental iron each day for 60 days, their take-home pay increased by 37%. The relevance of these findings was less than clear-cut because an improvement also occurred in anemic men given a placebo. The cash incentive that had been provided to encourage participation in the study and that enabled the placebo group to purchase additional food was believed to explain the improvement in both hematologic status and work performance. This conclusion was supported by follow-up studies 45 days after iron supplementation was terminated. The anemic men who improved with the placebo had again become anemic, with an attendant decline in work output, whereas those given the iron supplement maintained the improvement in both hematologic status and work performance. The design of the study permitted the benefit/cost ratios based on the predicted yearly increase in latex collected and the cost of iron administration. The calculated ratio of 260:1 underscores the economic importance of eradicating iron deficiency in developing countries.

Further support for the contention that the correction of iron deficiency enhances work output of the sustained endurance type was furnished by studies on a tea plantation in Sri Lanka.[32] As in the Indonesian study, work output could be measured precisely by weighing the quantity of tea collected each day. Compared with a placebo group, women given iron supplementation for 1 month picked significantly greater quantities of tea, and the degree of improvement in work output was directly related to the severity of the original anemia. An intriguing observation relating to voluntary physical activity was also made in this study. The level of activity during 24 hours was recorded electronically, using a small sensing device strapped to the ankle of each subject. Following iron therapy, a progressive increase in daily physical activity was observed. It became statistically significant during the third week of iron replacement. The improvement in physical activity may have resulted from alleviation of the symptoms of tiredness and weakness commonly attributed to iron deficiency.

Limitations in work performance have traditionally been attributed to the attendant anemia rather than to the metabolic consequences of tissue iron deficiency. The results of animal studies conducted by Finch and co-workers,[7] however, indicate that a deficit in tissue iron is also important. A treadmill was used to measure the work performance of normal and iron-deficient rats. The hemoglobin concentration in the two groups was adjusted to identical levels immediately prior to testing to eliminate the effects of anemia. The iron-deficient rats showed a marked impairment in running ability and a significant improvement in performance 24 hours after iron administration. The defect was fully corrected within 4 days, indicating that the metabolic abnormalities imposed by iron deficiency are rapidly reversible.

Published studies of work performance in a field setting do not distinguish between the consequences of anemia and tissue iron deficiency. A recent study in women athletes suggests, however, that the hemoglobin level is not the major deficit in chronic iron deficiency.[33] In this report, the effect of 2 weeks of iron therapy on exercise performance and exercise-induced lactate production was measured in trained athletes. Although performance measured on a bicycle ergometer was unchanged after iron therapy, blood lactate levels at maximum exercise decreased significantly from 10.3 to 8.4 mmol/L in the iron-deficient women following therapy ($P < .03$). These observations were interpreted as evidence that iron plays an important role in oxidative metabolism and that iron-depleted muscles call on anaerobic metabolism for energy needs, with greater lactate production. Dallman has emphasized the importance of differentiating brief intense activity that relates to oxygen delivery and the concentration of circulating hemoglobin from prolonged endurance tasks that depend to a greater extent on muscle oxidative capacity.[5] Further studies of work performance are needed to provide a clearer understanding of these separate deficits in persons with iron deficiency.

NEUROLOGICAL FUNCTION

Poor scholastic performance as well as chronic fatigue and other nonspecific symptoms have often been attributed to iron deficiency. Unfortunately, in many of the earlier studies, especially those in children, it has not been possible to distinguish the effects of iron deficiency from other factors such as nutritional and social deprivation.[34] Furthermore, tests used to evaluate mental development in children are relatively crude, making it difficult to isolate the component attributable to iron lack. Because symptoms in adults are largely subjective, it has been difficult to differentiate true responses to iron from placebo effects.

Despite these limitations, a pattern of age-related neurological dysfunction appears to be emerging from the results of several more recent carefully controlled investigations. Most of them involve children. This literature has been reviewed recently[34,35] and an exhaustive evaluation of all the related publications will not be given here; rather, an attempt will be made to develop the common theme that is evident in the newer studies. Only sensory and behavioral disturbances have been recorded consistently; motor function is usually normal. Moreover, several different observations point to a primary abnormality in the ability of iron-deficient children to direct attention to a specific task and suggest that other apparent neurological deficiencies may be a consequence of this primary neuropsychiatric dysfunction.

Most investigators have selected a relatively small age range to study. It is convenient to consider their findings in terms of four age groups: infants (0 to 2 years of age), preschool children (3 to 6 years of age), school children (6 to 12 years of age), and adults.

Infants. The assessment of mental development and performance in this age range is particularly challenging. The Bayley Scales of Infant Development (BSID) is the tool that has been used most widely to measure differences between iron-deficient infants with or without anemia and their iron-replete counterparts. The BSID gives a comprehensive and carefully standardized assessment of developmental status between the ages of 3 and 30 months of age.[36] It consists of three complementary parts: the mental development index (MDI), which evaluates sensory-perceptual acuity and discrimination; the motor scale, which provides an assessment of body control and coordination of both large and small muscle movements; and the behavior record (IBR), which is a measure of the infant's social and objective orientation to the immediate environment. Although the BSID is extremely useful, it is difficult to administer optimally and is heavily dependent on the experience of the examiner. Moreover, when comparisons between numerous elements of the test are made, the probability of finding a significant difference in isolated elements by chance alone is increased, especially when one considers the rapid developmental changes occurring during infancy.[37] There is also a lack of continuity in performance, demonstrated by a poor correlation between test and retest scores separated by months. Longitudinal comparisons between treated and untreated experimental groups must therefore be carried out in a short time that may be insufficient to demonstrate the corrective effects of iron administration.

Despite these methodological handicaps, impaired attention span and cognitive developmental abnormalities have been reported with surprising consistency in studies in which infants at or near 2 years of age have been included in the sample. Indeed, apparent discrepancies between different reports in the literature may be a reflection of the age group studied. Deinard and co-workers[38] used three tests, a visual habituation measure, the BSID, and the Uzigris and Hunt Ordinal Scales (I, II, IV) of Psychological Development, to evaluate nonanemic children aged 11 to 13 months with ferritin values of either <9 ng/mL or >20 ng/mL. This is a relatively small variation in iron status, and there were no differences in overall performance between the two groups. On the other hand, when 37 15-month-old infants were studied by Walter and colleagues,[39] values for the BSID MDI were found to be significantly lower in children with iron deficiency anemia than in carefully matched controls, although both groups fell within the normal range for American children. Oral iron therapy (3 to 4 mg iron per kilogram per day) returned the MDI for iron-deficient infants to control values in 10 to 15 days and was without effect on the control infants. An improvement in cooperativeness and attention span on the BSID was also noted. A similar rapid improvement in cognitive function has been reported by Oski and Honig[40] after iron-deficient anemic infants with a mean age of 16 months were given treatment of 5 to 8 days of intramuscular iron. Finally, Lotzoff and colleagues[41] demonstrated the presence of a relationship between age and the detectable effects of iron deficiency in a single study of infants aged 6 to 24 months. When the infants were analyzed in three groups (6 to 12 months, 13 to 18 months, and 19 to 24 months), a significant decrease in the MDI with iron deficiency occurred only in the oldest age group. Furthermore, there was a significant positive correlation between iron status and MDI in this latter group.

Although these observations suggest that detectable abnormalities of neurological functions related to iron deficiency appear only in later infancy, the explanation is not immediately evident. Several possible reasons have been put forward. It may be an artifact of the method of evaluation. The BSID shifts from an emphasis on motor development at 12 months to more verbal and cognitive skills at 24 months. Only sensory, cognitive, and behavioral abnormalities have been observed. With few exceptions, normal motor function has been reported. On the other hand, the duration of iron depletion or the stage of neurological development at which it occurs may be important. It is equally possible that other factors unrelated to iron nutrition may play a role.

Preschool children. Abnormalities similar to those reported in older infants have been observed in preschool children. Pollitt and co-workers[42] studied 15 children with mild iron deficiency and 15 controls aged 3 to 6 years. The two groups performed equally well on the Stanford-Binet Intelligence Test and an oddity learning task. Iron-deficient children took more trials to achieve the required level of proficiency in three discriminatory learning tasks, however. Nevertheless, when presented immediately with the reverse problem (the previously incorrect stimulus was now correct), the two groups showed no differences, demonstrating satisfactory short-term memory. On the other hand, there was a significant decrease in performance when a separate, simple, short-term memory test was administered de novo. The authors interpreted their findings as consistent with a deficit in directing attention rather than a true learning rate deficiency. Differences in discrimination learning but not those related to the short-term memory test disappeared after 4 to 6 months of iron therapy. This study is somewhat compromised by suboptimal documentation of iron status and reassignment of subjects between the control and study groups. Nevertheless, the observations are intriguing and similar to others recently reported from Guatemala.[43]

School children. Poor scholastic performance, disturbances in attention and perception, and unsatisfactory conduct were reported in early studies carried out by Webb and Oski[44,45] among 14-year-old high school students living in an economically deprived community in Philadelphia. Unfortunately, methodological problems and the possible contribution of other nutritional as well as concommitant social factors preclude conclusive inferences from these investigations. The most complete study was undertaken recently in Indonesia.[46] Seventy-eight 11-year-old children who met strict criteria for iron deficiency anemia were compared with 41 carefully matched nonanemic controls. A neurological evaluation was performed before and after the supervised administration of ferrous sulfate or placebo for 3 months. Once again, a defect in directing attention appeared to be the primary abnormality.

The Borden-Wisconsin test for concentration, a measure of attention maintenance, was significantly improved after iron administration in anemic children, but not in the group given placebo or in the nonanemic children given iron. Raven Progressive Matrices IQ Scores of anemic and nonanemic children were not significantly different. Scholastic performance as measured by a modified standard achievement test was significantly better in nonanemic children, however; iron therapy led to an appreciable improvement, although the performance of the anemic children was still significantly lower than that of the nonanemic controls at the end of 3 months. The authors again considered the primary abnormality to be one of directing attention with a secondary effect on scholastic achievement. The deficit in academic performances may require a >3-month period for complete correction Unfortunately, the possible long-term consequences of childhood iron deficiency on adult academic achievement are unknown.

Adults Clear evidence of an association between iron deficiency and psychological or cognitive function in adults has not been obtained, although Tucker and colleagues[47] found preliminary evidence for a positive correlation between serum ferritin concentration and verbal fluency as well as electroencephalographic asymmetry. Higher ferritin values were associated with poorer nonverbal auditory task performance, however. Chronic fatigue and other nonspecific symptoms were reported to respond to iron therapy in some persons in one study,[48] but other investigators have produced conflicting results.[49–52]

The only sensory disturbance that has been documented clearly in adults is a perversion of taste leading to the consumption of non-food items (pica). A commonly encountered form in the United States is pagophagia or compulsive ice eating.[52] Indeed, this was the only nonhematological manifestation of iron deficiency detected by Rector and co-workers[53] in seven patients in whom regular phlebotomy for polycythemia vera had produced chronic iron deficiency. The craving for ice appeared to be specific. It could not be attributed to stomatitis or glossitis and was not satisfied by cold liquids or ice cream. Pagophagia is rapidly cured by doses of iron less than those required to correct anemia or repair the iron-storage deficit.[3,53,54] Various forms of pica have been observed in several parts of the world. It occurs at all ages but is most frequently encountered in children and pregnant women.[3] Although pica appears to be a valid symptom of iron deficiency in many circumstances, several of the substances consumed adsorb iron and inhibit its absorption, thus contributing to the deficiency state.[3] Moreover, there may be other causes of pica itself. For example, ash eating is common among South African black women who are not often iron deficient.[55]

In summary, there is an increasing body of evidence connecting iron deficiency with or without anemia with deficits in attention span leading to learning and problem-solving difficulties in children. The neurophysiological mechanisms involved are still largely speculative. Several parts of the brain contain large quantities of iron,[56] and iron is required for the activity of enzymes such as tyrosine hydroxylase, tryptophan hydroxylase, and monoamine oxidase that are important to amine neurotransmitter metabolism. A series of studies in rats carried out by Youdim and colleagues[57] suggests that dopamine receptor function is impaired in iron deficiency and leads to the observed neuropsychological changes.

No information implicates academic or social handicaps in adults with iron deficiency during childhood or the later years, but it seems likely that impaired learning could have important consequences for the person's subsequent achievement in a world of increasing technological complexity. The insidious but potentially far-reaching consequences both for the individual and the community, particularly in developing countries where iron deficiency is still a prevalent nutritional disorder, should not be ignored. Although our understanding of the neuropsychological consequences of iron lack is undoubtedly fragmentary at this time, the studies outlined above provide ample reason for concern and a convincing rationale for programs to ensure adequate iron nutrition in the growing child.

CONCLUSIONS

The key nonhematologic liabilities of iron deficiency in humans relate to abnormalities in host defense, work performance, and neurological function. The relationship between iron status and infection remains highly controversial. A defect in cell-mediated immunity and neutrophil function can be readily demonstrated in iron-deficient patients and may predispose to common respiratory or diarrheal infections. On the other hand, evidence shows that vigorous iron therapy may enhance the risk of certain overwhelming infections, especially in the immunocompromised host. In several field trials, alleviation of iron deficiency has led to an increase in work output of the sustained endurance type, a finding that has important socioeconomic implications in developing countries. With regard to neurological function, the most important defect appears to be an impairment in attention span and cognitive development that has been demonstrated in late infancy, preschool, and school-age children. This defect in learning ability could have far-reaching consequences in areas of the world where iron deficiency remains a common nutritional problem.

REFERENCES

1. Fairbanks VF, Fahey JL, Beutler E: Clinical Disorders in Iron Metabolism. Orlando, Fla. Grune & Stratton, 1971, p 1

2. DeMaeyer E, Adiels-Tegman M: The prevalence of anaemia in the world. World Health Stat Q 38:302, 1985

3. Bothwell TH, Charlton RW, Cook JD, Finch CA: Iron Metabolism in Man Oxford, Blackwell Scientific, 1979, p 1

4. Beutler E, Fairbanks VF: The effects of iron deficiency, in Jacobs A, Worwood M (eds)· Iron in Biochemistry and Medicine. II. London, Academic Press, 1980, p 393

5. Dallman PR: Manifestations of iron deficiency. Semin Hematol 19:19, 1982

6. Jacobs A. Non-haematological effects of iron deficiency. Clin Haematol 14:353, 1982

7. Finch CA, Miller LR, Inamdar AR, Person R, Seiler K, Macker R. Iron deficiency in the rat. Physiological and biochemical studies of muscle dysfunction. J Clin Invest 58:447, 1976

8. Bullen JJ, Rogers HJ, Griffiths: Role of iron in bacterial infection. Curr Top Microbiol Immunol 80.1, 1978

9. Beisel WR. Single nutrients and immunity Am J Clin Nutr 35. 417, 1982 (suppl)

10. Weinburg ED· Iron withholding: A defense against infection and neoplasia. Physiol Rev 64:65, 1984

11. MacKay HM: Anaemia in infancy: Its prevalence and prevention. Arch Dis Child 3:117, 1928

12. Andelman MF, Sered BR: Utilization of dietary iron by term infants. Am J Dis Child 111:45, 1966

13. Burman D: Hemoglobin levels in normal infants aged 3 to 24 months, and the effect of iron. Arch Dis Child 47:261, 1972

14. Basta SS, Soekirman MS, Karyadi D, Scrimshaw NS: Iron deficiency anemia and the productivity of adult males in Indonesia. Am J Clin Nutr 32·916, 1979

15. Higgs JM, Wells RS: Chronic mucocutaneous candidiasis: Associated abnormalities of iron metabolism. Br J Dermatol 86:88, 1973 (suppl 8)

16. Vyas D, Chandra RK: Functional implications of iron deficiency, in Stekel A (ed): Iron Nutrition in Infancy and Childhood. New York, Raven Press, 1984, p 45

17. Joynson DHM, Jacobs A, Walker DM, Dolby AE: Defect of cell-mediated immunity in patients with iron-deficiency anaemia. Lancet 2:1058, 1972

18. Chandra RK, Saraya AK: Impaired immunocompetence associated with iron deficiency J Pediatr 86·899, 1975

19. Macdougall LG, Anderson R, McNab GM, Katz J The immune response in iron-deficient children: Impaired cellular defense mechanisms with altered humoral components. J Pediatr 86:833, 1975

20. Krantman HJ, Young SR, O'Donnell CM, Rachelefsky GS, Stiehm ER: Immune function in pure iron deficiency. Am J Dis Child 136:840, 1982

21. Chandra RK: Reduced bactericidal capacity of polymorphs in iron deficiency. Arch Dis Child 48:864, 1973

22 Sagone AL Jr, Balcerzak SP: Activity of iron-containing

enzymes in erythrocytes and granulocytes in thalassemia and iron deficiency. Am J Med Sci 259:350, 1970

23. Sussman M: Iron and infection, in Jacobs A, Worwood M (eds): Iron in Biochemistry and Medicine. London, Academic Press, 1974, p 649

24. Payne SM, Finkelstein RA The critical role of iron in host-bacterial interactions. J Clin Invest 61:1428, 1978

25. Barry DMJ, Reeve AW: Increased incidence of gram-negative neonatal sepsis with intramuscular iron administration. Pediatrics 60:908, 1977

26. Becroft DMO, Dix MR, Farmer K: Intramuscular iron-dextran and susceptibility of neonates to bacterial infections. Arch Dis Child 52:778, 1977

27 Masawe AEJ, Muindi JM, Swai GBR. Infections in iron deficiency and other types of anaemia in the tropics. Lancet 2.314, 1974

28. Murray MJ, Murray AB, Murray MB, Murray CJ: The adverse effect of iron repletion on the course of certain infections. Br Med J 2:1113, 1978

29. Fleming AF: Iron deficiency in the tropics. Clin Haematol 11:365, 1982

30. Viteri FE, Torun B: Anaemia and physical work capacity. Clin Haematol 3.609, 1974

31. Gardner GW, Edgerton BR, Senewiratne B, Barnard RJ, Ohira Y Physical work capacity and metabolic stress in subjects with iron deficiency anemia. Am J Clin Nutr 30:910, 1977

32. Edgerton VR, Gardner GW, Ohira Y, Gunawardena KA, Senewiratne B: Iron-deficiency anaemia and its effect on worker productivity and activity patterns. Br Med J 2:1546, 1979

33. Schoene RB, Escourrou P, Robertson HT, Nilson KL, Parsons JR, Smith NJ: Iron repletion decreases maximal exercise lactate concentrations in female athletes with minimal iron deficiency anemia. J Lab Clin Med 102 306, 1983

34. Pollitt E, Leibel RL: Iron deficiency and behavior. J Pediatr 88:372, 1976

35. Pollitt E, Leibel RL (eds): Iron Deficiency: Brain Biochemistry and Behavior. New York, Raven Press, 1982, p 1

36. Bayley N: Bayley Scales of Infant Development Manual New York, Psychological Corp, 1969, p 1

37. Pollitt E, Greenfield D, Leibel R: Significance of Bayley Scale score changes following iron therapy. J Pediatr 92:177, 1978

38 Deinard A, Gilbert A, Dodds M, Egeland B: Iron deficiency and behavioral deficits. Pediatrics 68:825, 1981

39. Walter T, Kovalskys J, Stekel A: Effect of mild iron deficiency on infant mental development. J Pediatr 10:519, 1983

40 Oski FA, Honig AS: The effects of therapy on the developmental scores of iron-deficient infants. J Pediatr 92.21, 1978

41. Lotzoff B, Brittenham GM, Viteri FE, Wolf AW, Urrutia JJ. Developmental deficits in iron-deficient infants. Effects of age and severity of iron lack. J Pediatr 101:948, 1982

42 Pollitt E, Leibel RL, Greenfield DB, Iron deficiency and cognitive test performance in preschool children. Nutr Behavior 1:137, 1983

43. Pollitt E, Saco-Pollitt C, Leibel RL, Viteri FE: Iron deficiency and behavioral development in infants and preschool children. Am J Clin Nutr 43:555, 1986

44. Webb TE, Oski FA: Iron deficiency anemia and scholastic achievement in young adolescents J Pediatr 82:827, 1973

45. Webb TE, Oski FA. Behavioral status of young adolescents with iron deficiency anemia. J Spec Educ 8:153, 1974

46. Soemantri AG, Pollitt E, Kim I: Iron deficiency anemia and educational achievement. Am J Clin Nutr 42:1221, 1985

47. Tucker DM, Sandstead HH, Penland JG, Dawson SL, Milne DB: Iron status and brain function. serum ferritin level associated with asymmetries of cortical electrophysiology and cognitive performance. Am J Clin Nutr 39:105, 1984

48. Beutler E, Larsh SE, Gurney CW: Iron therapy in chronically fatigued, nonanemic women: A double-blind study. Ann Intern Med 52:378, 1960

49. Wood MM, Elwood PC. Symptoms of iron deficiency anaemia. A community survey. Br J Prev Soc Med 20:117, 1966

50. Elwood PC, Waters WE, Greene WJW, Sweetnam P, Wood MM: Symptoms and circulating haemoglobin level. J Chronic Dis 21:615, 1969

51. Morrow JJ, Dagg JH, Goldberg A. A controlled trial of iron therapy in sideropenia. Scot Med J 13:78, 1968

52. Elwood PC, Wood MM: Effect of oral iron therapy on the symptoms of anaemia. Br J Prev Soc Med 20:172, 1966

53. Coltman CA Jr: Pagophagia and iron lack. JAMA 207:513, 1969

54. Rector WG, Jr, Fortuin NJ, Conley CL. Non-hematologic effects of chronic iron deficiency. A study of patients with polycythemia vera treated solely with venesections. Medicine 61:382, 1982

55 Sayers G, Lipschitz DA, Sayers M, Seftel HC, Bothwell TH, Charlton RW: The relationship between pica and iron nutrition in Johannesburg Bantu adults. S Afr J Nutr 48:53, 1974

56 Spatz H: Eisennachweis im Gehirn, besonders in Zentren des extrapyramidalen systems. ZFD Gesamte Neurol Psychiatr 77:261, 1922

57. Youdim MBH, Yehuda S, Ben-Shachar D, Askenazi R: Behavioral and brain biochemical changes in iron-deficient rats: The involvement of iron in dopamine receptor function, in Pollitt E, Leibel RL (eds): Iron Deficiency: Brain Biochemistry and Behavior, New York, Raven Press, 1982, p 39

Ca^{2+} and Phospholipid-Dependent Protein Kinase (Protein Kinase C) Activity Is Not Necessarily Required for Secretion by Human Neutrophils

By Kenneth J. Balazovich, James E. Smolen, and Laurence A. Boxer

Ca^{2+}-dependent and phospholipid-dependent protein kinase (PKC) is a receptor for and is activated by phorbol esters. This enzyme is reportedly involved in the mechanism of superoxide anion (O_2^-) production and the release of intracellular granule contents from human neutrophils. As previously reported by others, we found that >75% of the total cellular PKC activity existed in a soluble form in untreated neutrophils and that this activity was enhanced in a dose-dependent manner by phorbol 12-myristate 13-acetate (PMA) and by phorbol 12,13-dibutyrate (PDBu). Furthermore, mezerein, an analogue of PMA that is thought to be a competitive inhibitor, did not activate PKC, and on the contrary, inhibited PMA-stimulated activity in a dose-dependent manner. Pretreatment of intact neutrophils with PMA or PDBu caused the "translocation" of PKC activity to the insoluble cell fraction; PKC translocation was not detected after mezerein stimulation at any of the tested concentrations. Neither did mezerein cause an increase in intracellular Ca^{2-}, as monitored by Quin 2 fluorescence. Both phorbol esters and mezerein stimulated intact neutrophils to generate O_2^- and release lysosomal enzymes into the extracellular medium. Finally sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated key differences in the patterns of endogenous phosphoproteins of neutrophils stimulated with phorbol as compared with mezerein. We therefore suggest that PKC activation may not be the only pathway required to elicit neutrophil responses.

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HUMAN NEUTROPHILS are known to respond to both chemotactic and chemical stimuli by migration to the site of inflammation, releasing intracellular granule contents and generating superoxide anions (O_2^-).[1-12] In addition, there appears to be a marked increase in the phosphorylation of a limited number of cellular proteins when neutrophils are treated with these agents.[11,12] For example, it has been demonstrated that the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine, the tumor promoters phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), and the diterpene ester mezerein all stimulate these responses.[4,11,13-18] Phorbol esters are particularly useful as activators since they have been shown to bind directly to protein kinase C (PKC),[19-21] an enzyme that requires both Ca^{2+} and phospholipid, but not calmodulin, as co-factors.[22,23] PKC also binds to and is activated by 1,2-diacylglycerols, which are products of arachidonic acid metabolism in the neutrophil. Mezerein and both PMA and PDBu possess many structural and physiological similarities as tumor promoters,[24] but more relevant to the present study is the finding that although it is not a phorbol diester, mezerein has been shown to displace PDBu from its binding site specifically, in a manner consistent with competitive inhibition of PKC.[25-29]

Although there is strong circumstantial evidence for phorbol derivatives inducing critical cellular responses through PKC, the exact mechanisms for phorbol-induced O_2^- production, degranulation, and phosphorylation are not known. There is evidence that the phosphorylation of a specific set of cellular proteins is causally related to PMA-induced reactions. For example, the release of serotonin may be related to the phosphorylation of a 47,000 dalton protein in platelets.[30] In addition, the treatment of mast cells with PMA has been shown to induce the release of histamine and to stimulate the phosphorylation of specific cellular proteins.[31] More important, rabbit neutrophils have been shown to secrete lysozyme and to phosphorylate endogenous cellular proteins when treated with PMA.[32] In untreated neutrophils, most of the cellular PKC activity is found in soluble form; the remaining activity is associated with cellular membranes and the cytoskeleton.[33,34] After neutrophils are treated with phorbol esters, PKC activity is primarily associated with insoluble cellular material (eg, membranes), a phenomenon that has been termed "translocation" to describe a potential physical shift in PKC activity.[35-37] In the absence of unambiguous data, however, it is not known if PKC translocation actually represents a change in the physical location of the enzyme (from soluble to insoluble cellular components); an alteration in PKC activities in those locations is also possible, especially in light of evidence that suggests two classes of binding sites on PKC for (^{3}H)PDBu.[33] Although the mechanism of cellular activation involving PKC is poorly understood, it is currently hypothesized that PMA induces certain neutrophil functions, such as degranulation and O_2^- production, by directly activating PKC.[10]

This report concerns the potential role of PKC in mediating neutrophil activation. In brief, we confirmed previous results indicating that PMA and PDBu induce PKC activation, PKC translocation, degranulation and O_2^- generation.[3-7,20,33] Mezerein did not stimulate PKC activity and no PKC translocation was detected, but it did block PMA-stimulated PKC activation and translocation. Mezerein also induced degranulation and O_2^- production to levels comparable with those caused by the phorbol esters. Most important, mezerein stimulated the phosphorylation of a slightly different set of endogenous cellular proteins that either PMA or PDBu. In

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light of these data, there may be at least two mechanisms for stimulating degranulation and O_2^- production, one of which may involve PKC.

MATERIALS AND METHODS

Cells. Whole human blood was obtained by venipuncture from normal, healthy donors 20 to 55 years of age. Neutrophils were isolated from this blood by Ficoll-Hypaque centrifugation and dextran sedimentation, followed by hypotonic lysis to remove residual erythrocytes.[39,40] Cells were washed twice with Dulbecco's phosphate buffered saline (PBS) or Hank's buffer (pH 7.5) before use; cells were >95% neutrophils as assayed by differential histologic staining, and were consistently >97% viable as assayed by trypan blue exclusion.

Reagents. Mezerein was obtained from LC Services (Woburn, Mass). Electrophoretic reagents were obtained from Bio-Rad (Rockville Center, NY). All other reagents were obtained from Sigma (St Louis), unless otherwise specified. Phorbol esters and mezerein were dissolved in dimethylsulfoxide and diluted with water to obtain final working stock solutions.

Protein kinase C: cell stimulation and fractionation. Cells were washed once in PBS and then incubated at 10^7 cells/mL in Hank's buffer containing the appropriate concentration of stimulus for 5 minutes at 37 °C. The cells were then washed once in Hank's buffer to remove unbound stimulus. The final cell pellet was lysed by resuspension at 5×10^7 cells/mL in extraction buffer (50 mmol/L Tris-HCl, pH 7.5, 50 mmol/L of 2-mercaptoethanol, 1 mmol/L of phenylmethylsulfonylfluoride, 2 mmol/L of EGTA, and 0.05% Triton X-100) and sonicated on ice twice for 30 seconds each using a microprobe on a Fisher model 300 dismembranator. Cell sonicates were centrifuged for 5 minutes at 500 g (4 °C) to remove nuclei and unbroken cells, and then at 100,000 g for 60 minutes. Supernatant material, containing soluble PKC activity (soluble fraction), was removed. Pelleted material, containing cellular membranes, granules, and presumably most of the larger cytoskeletal elements (insoluble fraction), was brought to the original sonicate volume with extraction buffer and resuspended by sonication twice for 30 seconds each. Control cells were treated in an identical manner, but substituting the vehicle (eg, dimethylsulfoxide, <0.2%) for the drug.

Protein kinase C assay. PKC activity was assayed by modifications of previously published methods.[19,41] Optimal conditions were established for calcium, phospholipid, and sample concentrations resulting in linear rates of histone phosphorylation. Fifty microliters of cell sonicate, soluble, or insoluble material was added to 180 μL of a solution containing (final concentrations) 25 mmol/L Tris-HCl (pH 7.5), 10 mmol/L of $MgCl_2$, 18 μg/mL of phosphatidylserine, 160 μg/mL of histone (type IIIS), and 50 μmol/L of ATP (containing 10^6 cpm/25 μL of assay solution of [$\gamma - {}^{32}P$] ATP, Amersham). Some assays were conducted in the presence of phorbol, mezerein, inhibitors, or agents such as 40 mmol/L of NaF or 1 mmol/L of *p*-nitrophenylphosphate (Sigma Phosphatase Substrate 104), intended to retard phosphatase activity during the assay. In all cases, the assay was allowed to proceed for up to 15 minutes at 30 °C with constant agitation. The reaction was stopped by the addition of 1 mL of ice-cold 25% trichloroacetic acid (TCA) followed by 0.2 mg of bovine serum albumin. Protein was collected on 0.45-μm HAWP membrane filters (Millipore) and assayed for the incorporation of radionucleotide using 10 mL of Hydrofluor (National Diagnostics) in a Searle Isocap 300 liquid scintillation system. Under these assay conditions, PKC activity was linear with respect to sample concentration from 10^4 to 10^7 cell equivalents per tube and time (from 5 minutes to at least 60 minutes). All assays were performed in triplicate. Control experiments included vehicle alone (dimethylsulfoxide, <1%), boiled cellular material, incubation at 4 °C, or the removal of calcium, phospholipid, and activators; in all cases, control values were subtracted from experimental values to obtain relative PKC activities.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. We modified the procedure of Andrews and Babior slightly[12,42] to study the patterns of endogenous phosphoproteins in human neutrophils. Freshly isolated neutrophils (<4 hours after phlebotomy) were incubated in 35 × 100-mm Petri dishes at 10^7 cells/mL in PBS plus 0.45 mmol/L of Ca^{2+} and 0.25 mmol/L of Mg^{2+} for 30 minutes at 37 °C. After removal of nonadherent cells, the neutrophils were incubated for 90 minutes in 3 mL of a solution containing 6 mmol/L of HEPES/ 6 mmol/L of Tris-HCl (pH 7.5), 0.15 mmol/L of NaCl, 10 mmol/L of glucose, 5 mmol/L of KCl, 1 mmol/L of $MgCl_2$, 0.25 mmol/L of $CaCl_2$, and 0.25 mCi ^{32}Pi per dish (Amersham). The medium was then replaced with buffer containing a stimulus for 5 minutes, after which the reaction was stopped by the addition of ice-cold buffer, followed by a brief rinse with 10% TCA. Neutrophils were scraped from the dish in 3 mL of ice-cold Collection Buffer (0.25 mol/L of sucrose, 0.1 mol/L of NaF, 10 mmol/L EDTA, 0.1 mmol/L of PMSF, 0.1 mmol/L of Aprotinin and 0.1 mmol/L of trypsin inhibitor [Boehringer-Mannheim]). Total cellular protein was precipitated with 25% TCA and extracted with acetone. Final pellets were solubilized in 50 μl of sample buffer [62.5 mmol/L of Tris, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS plus bromphenol blue] and boiled for 3 minutes before freezing. 35 μg of solubilized protein[42a] per lane was subjected to electrophoresis on 10% polyacrylamide gels (160 × 180 mm, according to the method of Laemmli.[43] Autoradiography was performed at −70 °C for 72 hours using Kodak X-Omat AR film.

Enzyme release and O_2^- assays. Neutrophils were incubated at 10^6 cells/mL in Hank's buffer or PBS (pH 7.5) for 5 minutes at 37 °C with or without phorbol, mezerein, or inhibitors. At the end of the incubation period, cells were centrifuged at 1,000 g for 10 minutes at 4 °C, and the supernatant was removed. The supernatant material was then assayed for content of vitamin B_{12} binding protein[44] or lactate dehydrogenase (LDH).[45] O_2^- generation was monitored as superoxide dismutase-inhibitable cytochrome c reduction according to published endpoint assays.[46]

Quin 2 assay for intracellular calcium. Quin 2 loading and fluorescence measurements were performed according to the method of Korchak and co-workers,[47] with the exception that PBS plus 1 mmol/L of Mg^{2+} and 0.6 mmol/L of Ca^{2+} was used as the medium.

RESULTS

Stimulation of PKC activity by phorbol esters. To examine the effect of phorbol esters and mezerein on PKC activity in human neutrophils, cell homogenates were separated into soluble and insoluble fractions, each of which was assayed using histone protein as a PKC substrate. PKC activity was defined as the specific incorporation of ^{32}Pi into histone (present in excess in the assay) which was dependent on the presence of phospholipid and calcium. Under the conditions of our assay, PKC was dependent on phospholipid and calcium, with optimal activity at 0.5 mmol/L of added calcium and 18 μg/mL of phosphatidylserine. Background activity (ie, in the absence of phospholipid, calcium, and activators) was <30 pmol ^{32}P/10 min/10^6 cell equivalents. Basal PKC activity (in the absence of phorbol) was low, ~33 ± 25 pmol ^{32}P/10 min/10^6 cell equivalents (Table 1). When incubated with 100 nmol/L of PMA or PDBu, soluble

Table 1 Human Neutrophil Protein Kinase C Activity

	pmol ^{32}P/10 min/ 10^8 cell Eq
Soluble fraction	33 ± 25
Insoluble fraction	15 ± 12
Soluble + nmol/L of PMA	578 ± 50 (81%)
Insoluble + nmol/L of PMA	136 ± 40
Soluble + 100 nmol/L PDBu	390 ± 42 (75%)
Insoluble + 100 nmol/L of PDBu	132 ± 14
Soluble + 100 nmol/L of mezerein	42 ± 18
Insoluble + 100 nmol/L of mezerein	3 ± 9
Soluble + 2 μg/mL 1,2-diolein	413 ± 26 (81%)
Insoluble + 2 μg/mL 1,2-diolein	98 ± 18

PMA, phorbol 12-myristate 13-acetate, PDBu, phorbol 12,13-dibutyrate.

Isolated human neutrophils were sonicated and centrifuged at high speed to obtain soluble (supernatant) and insoluble (pellet) fractions. Aliquots of each fraction were then assayed for protein kinase C (PKC) activity by monitoring radioactive phosphate incorporated into histone, and using phorbol esters, mezerein, or 1,2-diolein as stimuli. Results were obtained from at least ten separate experiments, and each experiment was performed in triplicate, the data are means ± 1 SD for these experiments Numbers in parentheses indicate the percentage of total PKC activity in that fraction.

material contained >75% of the total PKC activity (Table 1). Neither whole neutrophils nor neutrophil sonicates (either with or without nuclei) had any significant PKC activity.

PKC activity stimulated by PMA or PDBu in the soluble fraction from untreated cells was dependent on the concentration of the phorbol esters (Fig 1) In contrast, mezerein, an analogue and competitive inhibitor of PMA and PDBu,[19,25-29] did not activate soluble PKC over the same concentration range (Fig 1). PMA and PDBu also activated PKC in the insoluble fraction (Table 1). Mezerein did not activate insoluble PKC above basal levels at any concentration tested (results not shown).

If neutrophils were treated with 10^{-6} mol/L of mezerein before lysis and the isolated soluble material was incubated with various concentrations of PDBu, nearly total inhibition of PDBu-dependent PKC activity was observed (Fig 2). Pretreatment with 10^{-9} mol/L of mezerein only partially blocked PDBu-induced PKC activity (Fig 2). Similar results were found with PMA (data not shown). These data suggest that mezerein blocked previously active PKC, perhaps by competing directly with the binding of PMA and PDBu, a possibility that is consistent with previously published results.[25-29]

PKC translocation and functional activation of neutrophils by phorbol derivatives. Because mezerein blocked the stimulation of PKC activity by PMA and PDBu, we looked at the effect of these agents on various neutrophil functions. To test the ability of phorbol esters and mezerein to induce PKC translocation, cells were pretreated with various concentrations of PMA, PDBu, or mezerein, lysed, and then separated into soluble and insoluble fractions. Under these conditions, translocation of PKC activity from the soluble to the insoluble fraction was observed at PDBu concentrations from 10^{-7} to 10^{-3} mol/L (Fig 3A), with a maximum at 10^{-4} mol/L. Higher drug concentrations resulted in cell lysis, as indicated by an increase in lactate dehydrogenase release from the cytoplasm (Fig 3, inset). Similar results were obtained in experiments with PMA (data not shown). PKC translocation was not detected in

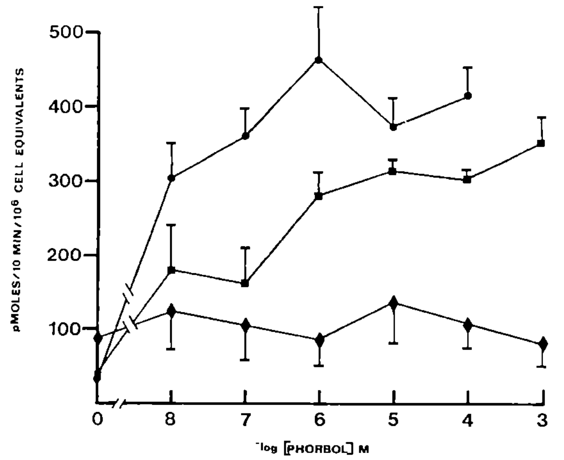

Fig 1. Phorbol ester- and mezerein-stimulated protein kinase C activity in human neutrophils. Cells were sonicated and centrifuged at 100,000 g to obtain soluble (supernatant) and insoluble (pellet) fractions. Soluble material was assayed for protein kinase C (PKC) activity in a cell-free assay for 10 minutes at 30 °C as described in the Materials and Methods section. Each point represents the mean (±1 SD) of at least ten experiments. The results show dose-response curves for phorbol 12-myristate 13-acetate (PMA) (●), phorbol 12,13-dibutyrate (PDBu) (■), and for mezerein (♦).

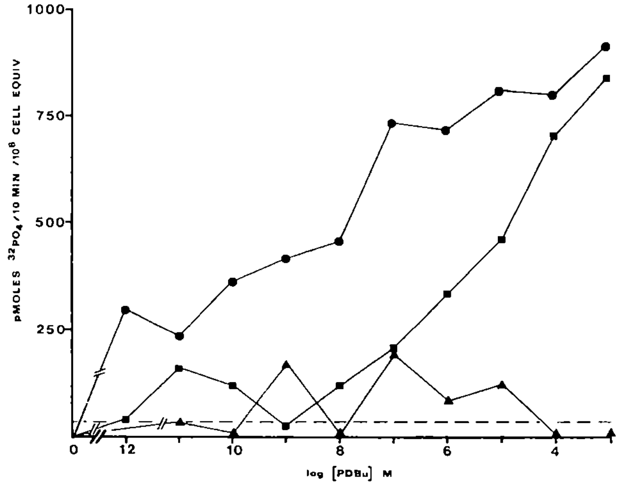

Fig 2. Inhibition by mezerein of phorbol 12,13-dibutyrate (PDBu)-stimulated protein kinase C activity Human neutrophils were incubated with buffer (●), 10^{-6} mol/L of mezerein (▲), or 10^{-9} mol/L of mezerein (■) for 5 minutes at 37 °C, washed to remove residual drug, and then sonicated. Sonicates were centrifuged at 100,000 g to obtain soluble (supernatant) and insoluble (pellet) fractions. The soluble fractions were then assayed for protein kinase C (PKC) activity using increasing doses of PDBu as a PKC activator in a cell-free assay. Each point represents the mean of two experiments, with each experiment performed in triplicate; SD from the mean were consistently <15%. The dotted line represents basal activity of PKC in the absence of phorbol.

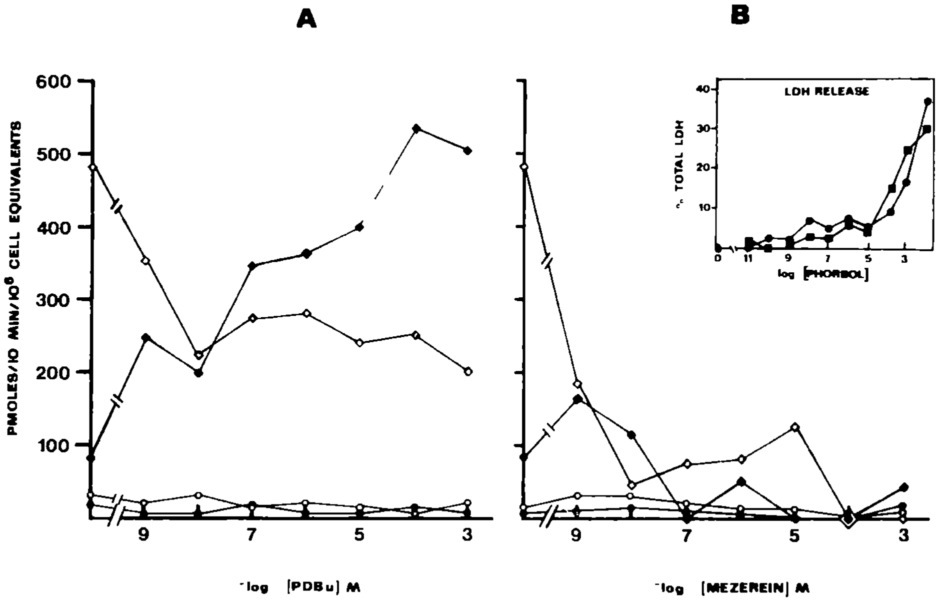

Fig 3. Effect of phorbol esters and mezerein on human neutrophil protein kinase C "translocation." Neutrophils were treated with various doses of either phorbol 12,13-dibutyrate (PDBu) (A) or mezerein (B) for 5 minutes at 37 °C and then sonicated on ice. Sonicates were then centrifuged at 100,000 g to obtain soluble (supernatant, ◇) and insoluble (pellet, ◆) fractions. Both fractions were then assayed for protein kinase C (PKC) activity using 2 μg/mL of 1,2-diacylglycerol as an activator (n = 5). Background kinase activity in soluble (○) and insoluble (●) fractions was measured in the absence of calcium, phospholipid, and activators. Inset: release of cellular LDH induced by exposure of neutrophils to PDBu (■) or mezerein (●).

mezerein-treated cells, and mezerein appeared to inhibit PKC activity (Fig 3B).

Phorbol esters and mezerein were tested for their ability to stimulate the release of vitamin B_{12} binding protein, a constituent of specific granules, from intact cells. PDBu stimulated the release of intracellular granule contents into the extracellular medium (Fig 4), with maximum degranulation of 63% obtained at 10^{-8} mol/L. Experiments using identical concentrations of PMA produced similar results (data not shown). Mezerein also induced up to 60% degranulation, but a maximal response was obtained at drug concentrations 3 to 4 orders of magnitude higher. These results were obtained without significant concomitant lactate dehydrogenase release $<10^{-4}$ mol/L (Fig 3, inset).

Human neutrophils were tested for their ability to generate O_2^- in response to phorbol esters and mezerein. Although mezerein proved to be a better stimulus for generating O_2^-, both mezerein and PDBu induced the production of O_2^- in a dose-dependent manner (Fig 5). Maximal production of O_2^- was 10 nmol/5 min/10^6 cells using 10^{-4} mol/L of PDBu or 10^{-7} mol/L of mezerein.

Our data suggested that mezerein stimulated neutrophils to degranulate and produce O_2^- through a mechanism that was independent of PKC, since no PKC translocation and little PKC activity was observed, and that was different from that stimulated by PMA and PDBu. Because PKC is activated by calcium and because phorbol esters decrease the K_m for calcium, we wanted to determine if mezerein produced its effects by changing intracellular calcium concentrations. Concentrations of 1μmol/L of this compound did not cause a rise in intracellular calcium (data not shown). *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), however, at a 0.1 μmol/L of concentration, caused the expected rise in intracellular calcium.[47] These data suggest that mezerein produces its cellular effect without causing a rise in intracellular calcium, much as has been reported for PMA and PDBu.

Phorbol ester-induced phosphorylation of endogenous cellular proteins. We performed an SDS-PAGE analysis of endogenous cellular phosphorylation patterns generated by treating cells with phorbol esters or mezerein in an attempt to understand how such different responses could be elicited by similar agents. Cells were metabolically labeled with radioactive phosphorus (ie, ^{32}Pi) and then incubated with 10^{-6} mol/L of PMA, PDBu, or mezerein for 5 minutes. Cellular protein was then collected and phosphorylation patterns were analyzed by separating 35 μg of protein by SDS-PAGE and visualizing phosphoproteins by autoradiography. The results of these experiments are presented in Fig 6. Control cells, which were incubated in the absence of phorbol or mezerein (Fig 6, D′), had at least 11 phosphoproteins visible by these methods. The high degree of phosphorylation observed in cells incubated without a stimulus was probably due to partial activation of control cells secondary to their adherence to Petri plates during phosphate loading. In all cases, treated cells had an increase in both the number of phosphoproteins (as compared with the control) and in the

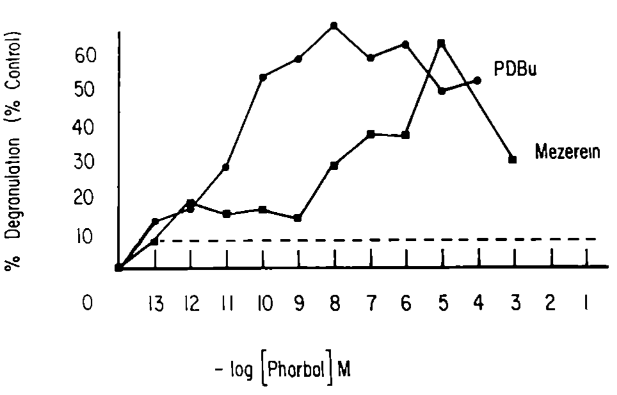

Fig 4. Phorbol ester- and mezerein-induced degranulation of human neutrophils. Neutrophils were incubated for 5 minutes at 37 °C with various concentrations of either phorbol dibutyrate (PDBu, ●) or mezerein (■). The incubation medium was then assayed for content of Vitamin B_{12} binding protein. Each point represents the mean of at least three experiments. The dotted line indicates basal degranulation (in the absence of activator).

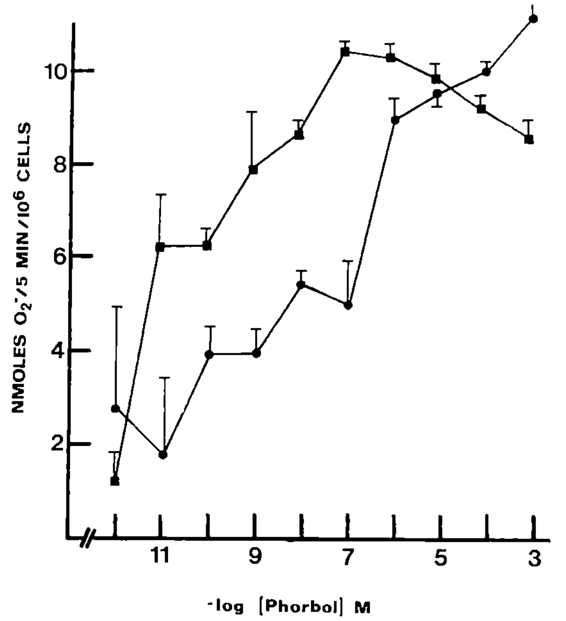

Fig 5. Effect of phorbol esters and mezerein on human neutrophil superoxide anion production. Neutrophils were incubated with various concentrations of either phorbol 12,13-dibutyrate (PDBu, ●) or mezerein (■) for 5 minutes at 37 °C. The incubation medium was then assayed for superoxide dismutase-inhibitable superoxide anion (O_2^-) production, monitored by the reduction of cytochrome c in the assay medium. Each point represents the mean ±1 SD of at least three experiments.

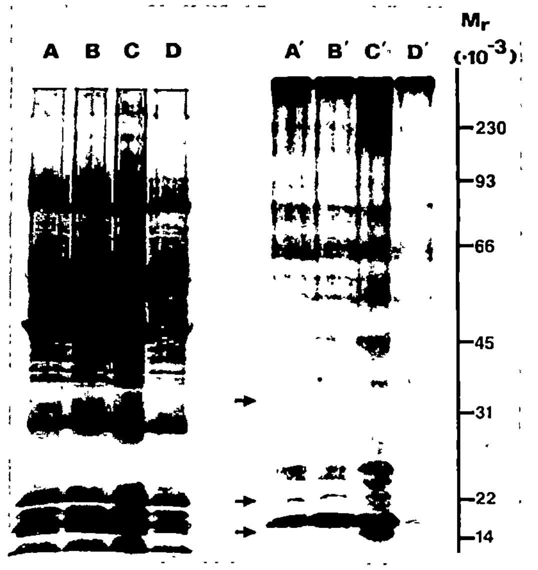

Fig 6. Endogenous phosphorylation patterns from neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), or mezerein. Neutrophils were treated with 10^{-6}-mol/L concentrations of phorbol esters or mezerein for 5 minutes at 37 °C, and the reaction was stopped by addition of ice-cold buffer. Total cellular protein was collected by trichloroacetic acid precipitation and acetone extraction. Phosphorylation patterns were visualized by the separation of 35 μg of protein[57] on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Autoradiography was performed at −70 °C for 72 hours using a Lightening Plus (DuPont) intensifying screen. Lanes A' through D' are stained with Coomassie brilliant blue for protein, and lanes A' through D' are the corresponding autoradiographs. A, PMA; B, PDBu; C, mezerein; D, control cells incubated in buffer plus vehicle (<0.1% dimethylsulfoxide). Numbers (right) represent the relative mol wts of protein standards run in parallel with lanes A through D. Arrows: additional labeling was observed at 32 and 14 kd, and a doublet was observed at 20 kd.

density of labeling of bands Phosphoprotein patterns for cells treated with PMA (Fig 6, A') and with PDBu (Fig 6, B'), both used at 100 ng/mL, were nearly identical. Cells treated with 10^{-6} mol/L of mezerein (Fig 6, D'), however, had a higher degree of phosphorylation of proteins at 45, 38, 25, 24, and 15 kd as compared with phorbol-treated cells. More important, additional labeling was observed at 32 and 14 kd, and a doublet was observed at 20 kd, (arrows, Fig 6), results that were not observed in phorbol-treated cells. Although none of the phosphoproteins generated by phorbol or mezerein treatment were identified in this study, these preliminary results suggest that mezerein-treated intact neutrophils phosphorylate a slightly different subset of cellular proteins than do phorbol-treated cells.

DISCUSSION

Tumor-promoting phorbol esters elicit a series of responses when used as stimuli for intact neutrophils.[1-17] For example, rabbit neutrophils release lysozyme from intracellular specific granules and also phosphorylate a limited number of cellular proteins when stimulated with PMA.[32] In addition, Andrews and Babior[12,42] and White and colleagues[32] showed that phorbol derivatives stimulate the phosphorylation of a limited number of endogenous cellular proteins in human and in rabbit neutrophils. Correlations between the presence of phorbol esters and protein phosphorylation have been demonstrated in fibroblasts,[48] mast cells,[31] platelets,[20] and lymphocytes,[35,36] as well as in neutrophils.[11,12,28,32,49] Although it is known that some phorbol esters bind directly to PKC, details of the activation mechanism after binding that contributes to these effects are not known.

We confirmed previous results demonstrating that PMA, PDBu, and mezerein all induce O_2^- production, degranulation and phosphorylation events in the human neutrophil. Mezerein stimulated higher levels of O_2^- production, though lesser amounts of degranulation, than did PDBu at similar concentrations. This observation compares favorably with that of Goldstein and co-workers[18] who demonstrated that mezerein at 10^{-8} to 1.5×10^{-7} was a better inducer of O_2^- production than was PMA.

We found that mezerein at 10^{-8} to 10^{-3} mol/L, unlike PMA and PDBu, induced no neutrophil PKC activity, nor did it lead to PKC translocation (Figs 2 and 3). Our results do not rule out the possibility that PKC had translocated to the insoluble fraction when treated with mezerein but was not active or was not detected under our assay conditions. For example, mezerein-activated PKC may not phosphorylate histone in our assay. Recent experiments have suggested this is not the case, since PMA and PDBu stimulated the phosphorylation of myosin light chain in our assay, but no such activity was detected at any concentration of mezerein tested (unpublished observations). We showed that mezerein blocked PDBu-stimulated PKC activity (Fig 3). In addition, whereas 10^{-6} mol/L of mezerein completely blocked both PMA- and PDBu-stimulated PKC activity both before and after cell disruption, 10^{-9} mol/L of mezerein only partially inhibited this event. These results are in direct contradiction to certain previously published reports involving mezerein.[29,50,51] For example, both crude[52] and partially purified[51] PKC from mouse brain cytosol gave comparable activities with either PMA (10^{-8} to 3×10^{-6} mol/L) or mezerein. In contrast, our results were obtained with crude soluble and insoluble fractions of neutrophils (Table 1 and Figs 1–3), and we consistently obtained inhibition of PKC activity using a wide range of concentrations. The contrary results[51,52] were reported only for narrow mezerein concentration ranges, although we demonstrated clear dose–response effects for concentrations from 10^{-8} to 10^{-4} mol/L. Although we have not directly shown that mezerein is a competitive inhibitor of PMA in our assay, our results demonstrate that pretreatment of whole cells with mezerein inhibits subsequent stimulation by PDBu (Fig 2) and are consistent with the hypothesis that mezerein and certain phorbol esters (ie, PMA and PDBu) may be competing for similar binding sites on the PKC molecule.[25–29] It is possible that mezerein has inhibited or activated cellular pathways other than those stimulated by PMA and PDBu, and that PKC inhibition is simply peripheral to the specific effect of this agent.

The important implication from our results is that PKC activity, as measured by commonly used techniques, can be dissociated from well-known and very important neutrophil functions, namely degranulation and O_2^- production, in the presence of mezerein. Significant differences were discovered in the phosphorylation patterns of endogeneous neutrophil proteins from cells treated with mezerein, a compound that may stimulate cellular functions through a different mechanism than that used by PMA and PDBu. It is also noteworthy that cellular activation by mezerein does not cause a rise in intracellular calcium as monitored by Quin-2 fluorescence. Our work provides additional evidence that it is possible to dissociate PKC activity, as monitored by histone phosphorylation, from certain other neutrophil functions. Intact neutrophils treated with either PMA or PDBu (in contrast to mezerein) exhibited both an activation of PKC and translocation of enzymatic activity from the soluble to the insoluble fraction. These results are in good agreement with those of Kraft and co-workers[35,36] and other researchers[17,33,53] who showed a similar shift in PKC activity. Although mezerein appeared to inhibit PKC translocation, further experimentation is clearly required to prove this point. In certain cases, translocation of PKC may be necessary to elicit subsequent events in the activation pathway, such as endocytosis, degranulation, and O_2^- production. For example, activation of PKC and its translocation to such cytoskeletal elements as microfilaments may trigger cell motility or surface protein redistribution, since the myosin light chain is phosphorylated by PKC.[54] Phosphorylation of the myosin light chain has been shown in at least one system to lead to some of these events.[55] PDBu has been shown to induce the phosphorylation of surface receptors for insulin[50] and for epidermal growth factor[48] and to induce the redistribution and capping of concanavalin A and Lyt-3 surface receptors in human lymphocytes.[56]

Our preliminary studies using mezerein suggest that PKC activity can be inhibited under conditions in which neutrophils are stimulated to degranulate and generate O_2^-. In addition, in this study, mezerein induced the phosphorylation of a slightly different subset of cellular proteins than either PMA or PDBu (Fig 6), a regulatory event which may or may not mediate the same secretory responses as the phorbols. Previous models have linked PKC-generated phosphorylation to later events in the stimulus–response pathway in neutrophils. In light of the present data, it is now necessary to reconsider the existing model to accommodate the possibility that a PKC-independent mechanism may be operating in activated neutrophils. Any proposed alternate mechanisms must be independent of increases in intracellular calcium, since mezerein did not cause a rise in Quin-2 fluorescence. It must also take into consideration the activity of kinases other than PKC, since there were clearly differences in the phosphoproteins generated by mezerein *v* phorbol. As a minimum, it must be noted that PKC activity, as monitored by the cell-free and endogenous protein phosphorylation assays, is not an absolute prerequisite for neutrophil stimulation.

REFERENCES

1 Kane SP, Peters TJ Analytical subcellular fractionation of human granulocytes with reference to the localization of vitamin B12-binding proteins Clin Sci Mol Med 49:171, 1975

2. Simon JD, Houck WE, Albala MA: Release of unsaturated vitamin B12 binding capacity from human granulocytes by the calcium ionophore A23187. Biochem Biophys Res Comm 73:444, 1978

3. Wright DH, Barlove DA, Gallin JI The differential mobilization of human neutrophil granules. Effects of phorbol myristate and ionophore A23187. Am J Pathol 87:273, 1977

4 Estensen RD, White JG, Holmes B: Specific degranulation of human polymorphonuclear leukocytes. Nature 248.347, 1974

5 Goldstein IM, Hoffstein ST, Weissmann G· Mechanism of lysosomal enzyme release from human polymorphonuclear leukocytes Effects of phorbol myristate acetate. J Cell Biol 66:647, 1975

6. Whitin JC, Chapman CE, Simons ER, Chovaniec ME, Cohen HJ: Correlation between membrane potential changes and superoxide production in human granulocytes stimulated by phorbol myristate acetate. J Biol Chem 255:1874, 1980

7. McPhail LC, Snyderman R. Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli Evidence that the same oxidase is activated by different transductional mechanisms. J Clin Invest 72:192, 1983

8. O'Flaherty JT, Dechatelet LR, McCall CE, Bass DA· Neutrophil aggregation: Evidence for a different mechanism of action by phorbol myristate acetate. Proc Soc Exp Biol Med 165.225, 1980

9. Mottala C, Romeo D: Calcium movement and membrane potential changes in the early phase of neutrophil activation by phorbol myristate acetate A study with ion selective electrodes J Cell Biol 93.129, 1982

10. Sha'afi RI, White JR, Molski TFP, Shefcyk J, Volpi M,

Naccache PH, Feinstein MB. Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in intracellular free Ca^{+2}. Biochem Biophys Res Comm 114:638, 1983

11. Schneider C, Zanetti M, Romeo D: Surface reactive stimuli selectively increase protein phosphorylation in human neutrophils. FEBS Lett 127·4, 1980

12 Andrews P, Babior B Endogenous protein phosphorylation by resting an activated human neutrophils Blood 61:333, 1983

13. Hatch GE, Gardner DE, Menzel DB: Chemiluminescence of phagocytic cells caused by *N*-formylmethionyl peptides J Exp Med 147:182, 1978

14 Lehmeyer JE, Synderman R, Johnston RB. Stimulation of neutrophil oxidative metabolism by chemotactic peptides: Influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristate acetate. Blood 54:35, 1979

15. Simchowitz L, Fishbein LB, Spilberg I, Atkinson JP. Induction of a transient elevation in intracellular levels of adenosine 3′, 5′-monophosphate by chemotactic factors, an early event in human neutrophil activation. J Immunol 124:1482, 1980

16. Petroski RJ, Naccache PH, Becker EL, Sha'afi RI Effect of chemotactic factors on calcium levels of rabbit neutrophils. Am J Physiol 237:243, 1979

17 Zigmond SH The ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factor. J Cell Biol 75·606, 1977

18. Goldstein BD, Witz G, Amoruso M, Stone DS, Troll W· Stimulation of human polymorphonuclear leukocyte superoxide anion radical production by tumor promoters. J Can Lett 11:257, 1981

19. Niedel JE, Kuhn LJ, Vandenbark GR Phorbol diester receptor copurifies with protein kinase C Proc Nat Acad Sci USA 80·36, 1983

20. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkiwa U, Nishizuka V: Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters J Biol Chem 2715·7841, 1982

21. Fujiki HJ, Tanaka Y, Miyake R, Kikkiwa U, Nishizuka Y, Sugimura T: Activation of calcium-activated, phospholipid-dependent protein kinase (protein kinase C) by new classes of tumor-promotors: Teleocidin and debromoaplysiatoxin. Biochem Biophys Res Commun 120.339, 1984

22. Takai Y, Kishimoto A, Iwasa Y, Kawahara Y, Marit J, Nishizuka Y. Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. J Biol Chem 254 3692, 1979

23. Brockelhurst KW, Hutton JHC: Involvement of protein kinase C in the phosphorylation of an insulin-granule membrane protein. Biochem J 220:283, 1984

24. Mufson RA, Fischer SM, Verma AK, Gleason GL, Slaga TJ, Boutwell RK: Effects of 12-*O*-tetradecanoylphorbol-13-acetate and mezerein on epidermal ornithine decarboxylase activity, isoproterenol-stimulated levels of cyclic adenosine 3′ 5′-monophosphate, and induction of mouse skin tumors in vivo Cancer Res 39:4791, 1979

25 Shoyub M, Todaro GJ: Specific high affinity membrane receptors for biologically active phorbol and ingenol esters Nature 288:451, 1980

26. Dunphy WG, Delclos KB, Blumberg PM. Characterization of specific binding of (^{3}H)phorbol 12,13-dibutyrate and (^{3}H)phorbol 12-myristate 13-acetate to mouse brain Cancer Res 40:3635, 1980

27. Delclos KG, Nogle DS, Blumberg PM Specific binding of phorbol ester tumor promoters to mouse skin Cell 19:1025, 1980

28. Goodwin BJ, Weinberg JG: Receptor-mediated modulation of human monocyte, neutrophil, lymphocyte and platelet function by phorbol diesters. J Clin Invest 70.699, 1982

29. Miyake R, Tanaka T, Tsuda T, Kaibuchi K, Kikkiwa U, Nishizuka Y Activation of protein kinase C by non-phorbol tumor promotor, mezerein. Biochem Biophys Res Commun 121 649, 1984

30 Imaoka I, Lynham JA, Haslam RJ· Purification and characterization of the 47,000 dalton protein phosphorylated during degranulation of human platelets. J Biol Chem 258:11404, 1983

31 Katakami Y, Kaibuchi Y, Sawamura M, Takai Y, Nishizuka Y: Synergistic action of protein kinase C and calcium for histamine release from rat peritoneal mast cells Biochem Biophys Res Commun 121·573, 1984

32. White JR, Huang CK, Hill JM, Naccache PH, Becker EL, Sha'afi RI: Effect of phorbol 12-myristate 12-acetate and its analogue 4 alpha phorbol 12, 13-didecanoate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils. J Biol Chem 259·8605, 1984

33 Wolfson M, McPhail LC, Nasrallah VW, Synderman R: Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: Potential role in the activation of the respiratory burst enzyme. J Immunol 135:2057, 1985

34. Balazovich KJ, Curnutte JC, Petrequin P, Smolen JE, Boxer LA· An inhibitor of protein kinase C may be associated with specific granules of human peripheral blood neutrophils Clin Res 33.5430, 1985

35. Kraft AS, Anderson WB, Cooper HL, Sando JJ: Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells J Biol Chem 257·13193, 1982

36. Kraft AS, Anderson WB: Phorbol esters increase the amount of Ca^{+2} and phospholipid-dependent protein kinase associated with plasma membrane. Nature 301:621, 1983

37. Wooten MW, Wrenn RW: Phorbol ester induces intracellular translocation of phospholipid/Ca^{+2}-dependent protein kinase and stimulated amylase secretion in isolated pancreatic acini. FEBS Lett 171 183, 1984

38. Horowitz AD, Greenbaum E, Weinstein IB: Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of inhibitors of receptor binding in biological fluids Proc Nat Acad Sci USA 78:2315, 1981

39. Zabucci G, Romeo D: The dissociation of exocytosis and respiratory stimulation in leukocytes by ionophores. Biochem J 156.209, 1976

40. Boyum KA· Isolation of mononuclear cells and granulocytes from human blood Scand J Clin Lab Invest 21·77, 1968 (suppl 97)

41 Kuo JF, Anderson RGG, Wise BC, Macherlova L, Solomonsson I, Brackett NL, Katoh N, Shoji M, Wrenn RW: Calcium-dependent protein kinase: Widespread occurrence in various tissue and phyla of the animal kingdom and comparison of effects of phorbol esters Proc Nat Acad Sci USA 77.7039, 1980

42 Andrews PC, Babior BM. Phosphorylation of cytosolic proteins by resting and activated human neutrophils. Blood 61 333, 1984

42a. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72.248, 1976

43. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature Lond 227 680, 1970

44 Gottlieb C, Lau KS, Wasserman LR, Herbert V: Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B12 binding capacity antibody to IF, and serum unsaturated B12 binding capacity. Blood 25·875, 1965

45 Wacker WEC, Ulmer DD, Valee BL. Metalloenzymes and myocardial infarction II. Malic and lactic dehydrogenase activities and zinc concentrations in serum. N Engl J Med 255:449, 1956

46 Goldstein IM, Cerquiera M, Lind S, Kaplan HB. Evidence that the superoxide generating system of human leukocytes is associated with the cell surface J Clin Invest 59.249, 1977

47. Korchak HM, Vienne K, Rutherford LE, Wilkenfeld C, Finkelstein MC, Weissmann GB: Stimulus-response coupling in the

human neutrophil. II. Temporal analysis of changes in cytosolic calcium and calcium influx. J Biol Chem 259·4076, 1984

48. Jacobs S, Sahyoun NE, Saltiel AR, Cuatrecasa P· Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C. Proc Natl Acad Sci USA 80:6211, 1983

49. Okamura N, Ohashi S, Nagahishi N, Ishibashi S: Changes in protein phosphorylation in guinea pig polymorphonuclear leukocytes by treatment with membrane-perturbing agents which stimulate superoxide anion production Arch Biochem Biophys 228:270, 1984

50. Courturier A, Bazger S, Castagna M Further characterization of tumor-promoter-mediated activation of protein kinase C Biochem Biophys Res Commun 121 448, 1984

51. Leach KL, Blumberg PM Modulation of protein kinase C activity and (^{3}H) phorbol 12, 13-dibutyrate binding by various tumor promoters in mouse brain cytosol. Cancer Res 45:1958, 1985

52. Miyake R, Tanaka Y, Tsuda T, Kaibuchi K, Kikkiwa U, Nishizuko Y: Activation of protein kinase C by non-phorbol tumor promoter, mezerein. Biochem Biophys Res Commun 121:649, 1984

53 Drust DS, Martin TFJ: Protein kinase C translocates from membrane to cytosol upon hormone activation· Effects of thyrotropin-releasing hormone in GH3 cells. Biochem Biophys Res Commun 128:531, 1985

54 Naka M, Nishikawa M, Adelstein RS, Hidaka H: Phorbol ester-induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chains. Nature 306:490, 1983

55. Bourguignon LYW, Nagpal ML, Balazovich KJ, Guerriero V, Means AR: Association of myosin light chain kinase with lymphocyte membrane–cytoskeleton complex. J Cell Biol 95:793, 1982

56. McCaffrey PG, Friedman BA, Rosner MR; Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor J Biol Chem 259:12302, 1984

Antilymphocytic Antibodies and Marrow Transplantation. VIII. Recipient Conditioning with Clq-Affine Monoclonal Anti-Pan T Antibodies Prevents GVHD in Homozygous Fully Mismatched Mice

By Stefan Thierfelder, Udo Kummer, Rolf Schuh, and Josef Mysliwietz

An approach to suppressing secondary disease with antibodies was studied that differed from conventional antibody treatment of donor marrow in vitro. It consisted of (a) the selection of anti-Thy-1 antibodies with high affinity for Clq, the first subunit of the complement cascade, and (b) a single injection of such antibodies into prospective irradiated marrow recipients. Monoclonal mouse IgM and rat IgG 2c antibodies of high titers in complement-dependent test systems but with low affinity for Clq caused little immunosuppression. Monoclonal rat IgG2b or mouse IgG2a anti-Thy-1 antibodies with high affinity for Clq prevented acute and chronic mortality of graft-*v*-host disease (GVHD), however, when injected in irradiated CBA or AKR mice prior to C57BL/6 spleen and/or bone marrow cell transfusion. This treatment simultaneously suppressed residual host-*v*-graft reactivity of the irradiated mice, so that permanent hematopoietic engraftment ensued even at 5 or 6 Gy. Full chimerism and specific tolerance were obtained. Primary immune response to SRBC was clearly depressed in the chimeras; secondary immune response was not. Clearance of T cell antibody activity (>6 days), timing, and dose of injected antibody, as well as other modalities of the conditioning treatment that may have contributed to the remarkable immunosuppression, are discussed.

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EVIDENCE SHOWS that many monoclonal anti-T lymphocyte antibodies show little immunosuppression in mice even though they fix heterologous complement and lyse T cells up to high antibody dilutions in vitro.[1] Such antibodies suppress graft-*v*-host disease (GVHD) when used for in vitro pretreatment of donor marrow, provided lytic complement was added.[2,3] Polyclonal rabbit antibodies that react with T cells did not show this dependence on complement in the pretreatment of donor cells for suppression of GVHD in mice and dogs.[4-6] On the other hand little clinical suppression of GVHD is observed following in vitro treatment, in the absence of complement,[7] with monoclonal anti-human T cell antibodies. We recently found a class of rat monoclonal antibodies (mAb) against the pan T antigen Thy-1 that suppresses GVHD[8,9] without the addition of complement during in vitro treatment of donor marrow. They were of the rat IgG2b isotype and had previously been found to delay the rejection of tumor or skin allografts.[10,11] Analysis of the mechanism(s) that might promote T cell inhibition by these antibodies led to the conclusion that their comparatively high affinity for Clq, the first subunit of the complement cascade, facilitated the clearance of antibody-coated T cells in the marrow recipient.[12] Exploiting the capacity of Clq-affine anti-Thy-1 mAbs to inhibit T cells in vivo, we have now been able to show that a single injection of rat IgG2b anti-Thy-1 mAbs not only prevents GVHD of subsequently transfused fully mismatched spleen and/or bone marrow cells but also reduces residual host-*v*-graft reactions. This resulted in hematopoietic engraftment and lasting chimerism even after a dose of only 5 or 6 Gy total body irradiation. Finally, we report a mouse anti-Thy-1 mAb of IgG2a isotype with high affinity for Clq and the same immunosuppressive qualities that we observed in the rat IgG2b anti-Thy-1 mAb, namely suppression of GVHD either by pretreatment of donor cells even without complement or by treatment of irradiated prospective marrow recipients.

MATERIALS AND METHODS

Bone marrow transplantation. Twelve-week-old mice of the CBA and AKR (H-2^k, Ia^k) and C57BL/6 (H-2^b, Ia^b) strains, originally from the Jackson Laboratory (Bar Harbor, Me) and their (C57BL/6xCBA)F1 hybrids, were raised under specific pathogens-free (SPF) conditions in our institute A total of 5 × 10^7 spleen and 2 × 10^7 bone marrow cells (in one series of experiments, a tenth of this amount) from C57BL/6 mice were suspended in RPMI 1640, filtered through nylon mesh, pelleted, and resuspended. No attempt was made to separate RBCs from splenocytes. Spleen cells (0.25 mL) were injected together with bone marrow cells into the tail vein of CBA or AKR mice weighing 22 to 25 g, that had been exposed 24 hours earlier in transparent, thermoplastic acrylic resin containers to 4 to 8 5 Gy using ^{137}Cs (Gammacell, HWM-D-2000, Ottawa, Canada) at 131 rad per minute from opposing sources, with each target distance 35 cm. The animals were caged in groups of six, and the cages were changed twice weekly All mice were fed a fat-supplemented diet and antibiotic-supplemented water ad libitum They were weighed weekly and examined for clinical signs of GVHD

Typing Tests for chimerism were conducted on lymph node cells, using polyclonal antisera (CBA-anti-C57BL/6 and C57BL/6-anti-CBA) with a titer of 1:500 in an in vitro complement-dependent microcytotoxicity assay.[13]

Immunocompetence of chimeras. Specific donor-type skin-graft tolerance and humoral immune response to sheep RBCs (SRBCs) were evaluated. Tail skin grafts of C57BL/6 or Balb/c mice were placed on the lateral thoracic wall of allogenic mice. They were covered with vaseline-impregnated gauze and adhesive tape. After 10 to 14 days, the grafts were inspected every second day and later every week for signs of rejection The immune response to SRBCs was tested 4 days after primary or secondary immunization, using a method originally described by Jerne and Nordin.[14] Direct hemolytic plaques (IgM response) were formed following addition of diluted guinea pig complement. Further incubation followed at 37 °C for 1 hour. Indirect plaques (IgM + IgG response) were detected with anti-mouse Ig.[15] SRBC-agglutinating activity of mouse sera was tested in microliter plates using a 5% cell suspension.

Conditioning for suppression of GVHD with antibodies. Antibody (0.25 mL) was injected as indicated, usually 4 hours after total body irradiation, ie, 20 hours before transplantation, intraperitone-

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ally (i.p.) or subcutaneously (s.c.). For the incubation approach, donor cells were incubated for 1 hour at 37 °C with antibody and fresh nontoxic rabbit serum (1:6) in 0.25 mL final volume before transplantation. The cells were not washed.

Antibodies. RmT$_1$ and RmT$_2$ (Table 1) were selected from a fusion of rat myeloma IR 983 F[16] and Lou/c rat spleen cells immunized against Balb/c thymocytes. They were used as ascitic fluid with an antibody activity corresponding to 2 mg antibody purified by means of a preparative block electrophoresis. The titers of the antibody doses are listed in Table 1. HO-13-4 was bought as NEI-001 from New England Nuclear, Boston, and later produced from the hybridoma[17] obtained through ATCC (TIB 99). 63-002, Clone TC[18] was bought in the form of ascitic fluid from Bayer-Diagnostik, Munich, FRG.

Antibody test systems. Complement-dependent microcytotoxicity[13] was assayed in vitro using nontoxic rabbit serum as the complement source. Titer endpoint was 50% of unstained thymocytes.

Quantitative complement fixation was adapted as a micromethod to determine binding to membrane antigens.[19] Two units of guinea pig complement, causing 50% hemolysis, was added to 10 μL of serial dilutions of antibody and 100 μL of lymphocytes (10^7 cells/mL). Complement-fixing antibody titer was defined as the antibody dilution that in the presence of T cells caused 50% lysis of the hemolytic indicator system consisting of SRBCs sensitized with rabbit hemolysin.

Preparation of microtiter plates for enzyme-linked immunoassay (ELISA). For ELISA, polystyrene Nunc-Immunoplates (Nunc 439454, Wiesbaden, FRG) were treated with 50 μL per well of poly-L-lysine (PLL-Sigma P1274, Deisenhofen, FRG) at a concentration of 10 μg/mL in PBS. The plates were incubated overnight at 4 °C and then washed twice in PBS; 50 μL cell suspension of the required concentration was added to each well. The plates were incubated for 45 minutes at 4 °C and fixed with glutaraldehyde (GDA Sigma G5882) at a final concentration of 0.05% in PBS for 15 minutes at room temperature. The GDA solution was discarded, and the plates were washed three times with PBS; 50 μL per well of 0.15 mol/L of glycine was then added for 15 to 30 minutes. The glycine solution was subsequently discarded, and nonspecific binding sites were blocked by the addition of 100 μL per well of 5% inactivated mouse or rat serum, 1% BSA in PBS. The plates were sealed and stored in the dark at 4 °C.

The ELISA procedure was as follows. The blocking solution was removed from the plates, and the wells were washed twice with PBS prior to the addition of the primary antibody. Fifty microliters of the serial dilution of primary antibody (Table 1) in PBS containing 1% serum from the species of secondary antibody and 0.2% bovine serum albumin (BSA) was added to each well. The plates were incubated for 1 hour at room temperature and then subjected to six washing steps with PBS. To each well was added 50 μL of an appropriate dilution of secondary antibody mouse anti-rat or goat anti-mouse IgG conjugated with horseradish peroxidase (Jackson 212-3562, 415-3562, Avondale, Penn). The plates were incubated for 1 hour at room temperature and then washed six times with PBS. One hundred microliters of a substrate solution (1,2-phenylene diamine dihydrochloride (Fluka 78440, Neu-Ulm, FRG) 1 mg/mL of 0.1 mol/L of Tris-citrate pH 5.0 buffer and 0.01% H_2O_2 were added, and the plates were then incubated at room temperature in the dark for 30 minutes. The absorbance was determined at 405 nm. Titer was defined as 50% of maximal absorbance.

Clq uptake. All tests were carried out with human Clq. The use of readily available purified human Clq instead of its murine counterpart is justified by the fact that Clq is interchangeable between species (reviewed in ref. 20). This is supported by our observation that when tested for binding in a radioimmunoassay, fresh mouse serum totally inhibited subsequent uptake of biologically active purified human Clq: mouse thymocytes incubated with rat IgG2b anti-Thy-1 antibody could be prevented from binding ^{3}H-labeled human Clq by addition of fresh mouse serum. Finally, mouse and human serum could be exchanged as a source of Clq in an immunohistochemical technique in which we measured Clq retention on thymocytes first saturated with anti-Thy-1 and then incubated with peroxidase-labeled rabbit anti-human Clq (G. Hoffmann-Fezer, Munich).

Table 1. Titers of Monoclonal Rat and Mouse Anti-Thy-1 Antibodies used for Suppression of GVHD

			Log$_2$ Antibody Titers in		
mAb	Species	Isotype	Antigen* Binding	Microcytotoxicity	C′ Fixation
RmT1	Rat	IgG2b	16	16	14
RmT2	Rat	IgG2c	16	10	8
HO-13-4	Mouse	IgM	15	20	14
63-002	Mouse	IgG2a	12	14	6

mAb, monoclonal antibody.

*C57BL/6 thymocytes were used throughout; antigen binding was determined in enzyme-linked immunosorbent assay.

For the radioimmunoassay, all experiments were carried out at 37 °C in BBS (0.15 mol/L of NaCl, 10 mmol/L of barbital HCl buffer, pH 7.3, 1 mmol/L of $CaCl_2$, 0.5 mmol/L of $MgCl_2$) containing 20% heat-inactivated mouse serum (ultracentrifuged prior to use) and 0.1% sodium azide in 96-well, U-shaped microtiter plates in a total volume of 100 μL. Saturating concentrations of antibody were incubated with 5×10^5 viable thymocytes. Dilutions of tritiated human Clq prepared according to the method of Zubler and co-workers[21] and tritiated to a specific activity of ~40 Ci/nmol[22] were then added. Following first and second incubation (each 45 minutes), repeated washing steps with ice-cold BBS containing 1% heat-inactivated mouse serum and 0.1% sodium azide were carried out, and cells were collected by means of a cell harvester on glass fiber filters. The resultant cell-bound radioactivity was then determined by counting in a liquid scintillation spectrometer.

RESULTS

Antibody titers. Table 1 summarizes results of the simultaneous titration of antibodies used to suppress GVHD. Thymocytes were used as target cells. The antibodies titrated also against lymph node T cells, were about two dilutions lower (data not shown), which is explained by the lower Thy-1 antigen density on postthymic T cells.[23] Either no titers or very low ones were obtained in the C′-dependent test systems in the presence of fresh mouse serum. Attempts to modify the microcytotoxic test to enhance T cell lysis by mouse complement, ie, in the presence of fresh serum from certain mouse strains,[26] were not undertaken. In summary, our titrations reveal four antibodies potent in antigen binding and activation of heterologous C′.

Clq uptake. Contrary to the above test systems, our tritiated Clq test clearly separates the four antibodies into two groups according to their Clq-binding capacity (Fig 1). Although it is unusual that RmT$_2$ and HO-13-4, with high titers in C′-dependent tests, fail to bind Clq, we explain this discrepancy by the wash-dilution step that is necessary in the Clq test but not in the cytotoxic and the C′ fixation tests. Because the uptake of Clq was measured after the wash-dilution step necessary to remove unbound labeled Clq, we

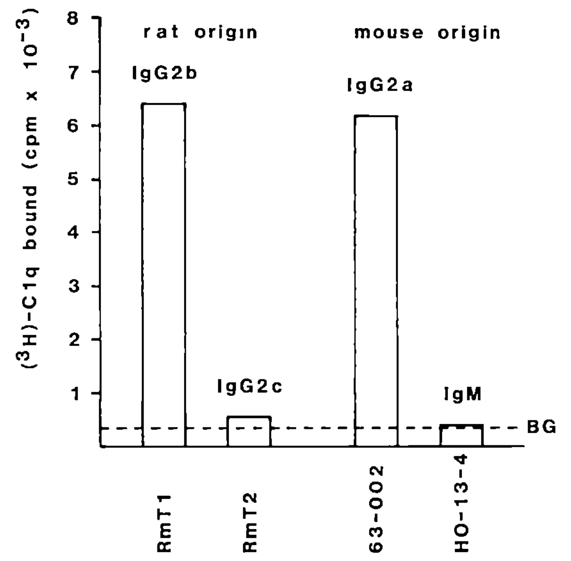

Fig 1. Binding of ^{3}H-labeled human Clq on murine C57BL/6 thymocytes saturated with rat anti-Thy-1 or mouse anti-Thy-1.2 monoclonal antibodies (mAbs). BG, background values without antibody.

actually measured the retention of Clq by thymocytes saturated with antibody following wash-dilution (binding titer shown in Table 1). Antibodies that fail to retain Clq but still activate C′ were regarded as Thy-1 antibodies with low Clq-binding affinity, ie, a Clq affinity that does not withstand the wash-dilution of the Clq uptake test but is good enough for C′ activation in C′-dependent cytotoxicity and C′ fixation.

As explained in the Materials and Methods section, Clq from human or mouse serum is interchangeable. The differences in uptake of Clq that were found with the readily available purified human Clq were observed indirectly also for the Clq equivalent in mouse serum. In summary, Clq uptake made it possible to group anti-Thy-1 antibodies into a rat IgG2b and mouse IgG2a isotype with high affinity and a rat IgG2c and mouse IgM isotype with low Clq affinity. Furthermore, Clq uptake can be estimated using both fresh heterologous and homologous mouse complement.

Circulating antibody activity. Injection of 0.25 mL of antibody leads to comparable antibody titers in the serum of mice 24 hours later whether it was administered s.c. or i.p. Even the titer of the s.c. administered IgM antibody (HO-13-4) was not lower than that administered i.p. Figure 2 shows that there was a high titer as early as 6 hours postinjection and a 3- to 4-$\log_2$ titer reduction on day 3. Mice irradiated with 8.5 Gy and injected 24 hours later had low antibody activity and nonirradiated mice a barely measurable antibody activity on day 6. Whether mouse antibodies against the rat anti-Thy-1 mAbs or whether the low absorption capacity of the irradiated aplastic mice was responsible for this difference in titer was not determined.

Donor cell pretreatment As shown in Table 2, HO-13-4 mouse IgM and RmT$_2$ rat IgG2c anti-Thy-1 suppressed acute GVHD mortality only in the presence of complement, whereas the other two antibodies, with high affinity for Clq, suppressed GVHD in the marrow recipient without requiring in vitro complement.

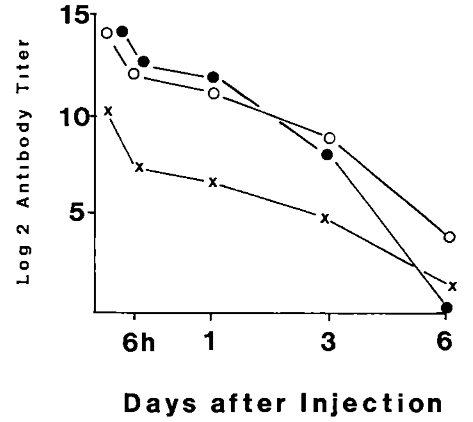

Fig 2. Clearance of lymphocytotoxic antibody activity in the serum of mice injected with 0.25 mL of anti-Thy-1 monoclonal antibodies (mAbs). RmT$_1$ (O,●), RmT$_2$ (x); mice irradiated with 8.5 Gy (O), nonirradiated mice (●,x). Each point represents pooled serum of three mice.

Conditioning of the recipient. Timing of antibody administration was important. Injection of RmT$_1$ into irradiated mice 20 hours or 4 hours before transplantation largely prevented acute and chronic GVHD mortality in CBA and AKR mice (Table 3, tests 1, 1b, 2, 2b, and 7). The antibody had lost most of its immunosuppressive effect when injected 2 days after transplantation, however (Table 3, test 4). Dose–response experiments likewise showed complete loss of immunosuppressive activity at an antibody dilution of 1:20 (Table 3, test 6) Prevention of GVHD was also possible with 63-002 mouse IgG2a anti-Thy-1, whereas conditioning with the two antibodies with low Clq affinity caused only a marginal delay in GVHD mortality (Figs 3 and 4). Chronic secondary disease induced by transfer of bone marrow without spleen cells was well suppressed with RmT$_1$ (Fig 3).

Chimerism. A reduction in our usual C57BL/6 cell numbers to 10%, ie, 5 × 10^6 spleen and 2 × 10^6 bone marrow cells, was still compatible with chimerism and prevention of GVHD in CBA mice irradiated with 8.5 Gy and conditioned

Table 2. Suppression of Acute and Chronic Mortality From GVHD* With Mouse Anti-Thy-1.2 or Rat Anti-Thy-1 Monoclonal Antibodies: Effect of Adding C′ In Vitro

				Survival (%)	
Antibody†	Source	Isotype	C′‡	Day 30	Day 60
HO-13-4	Mouse	IgM	+	100	66
	Mouse	IgM	−	0	
63-002	Mouse	IgG2a	+	100	83
	Mouse	IgG2a	−	100	66
RmT1	Rat	IgG2b	+	100	83
	Rat	IgG2b	−	100	100
RmT2	Rat	IgG2c	+	83	50
	Rat	IgG2c	−	0	

*Test system consisted of C57BL/6 spleen and bone marrow cells into CBA irradiated with 8.5 Gy (six mice per group)

†Diluted in 0.25 PBS with antibody titer as indicated in Table 1

‡Fresh nontoxic rabbit serum 1:6.

Table 3. Timing of Antibody Injection and Antibody Concentration in the Conditioning of Marrow Recipients With RmT1 Rat IgG2b Anti-Thy-1 for Suppression of GVHD

	Transplantation		Antibody Treatment		Survival (%)†		
Test No	Donor*	Recipient	Concentration (mg)	Timing‡ (h)	Day 30	Day 60	Day 100
1	C57BL/6	CBA	2	4	100	100	100
1b†	C57BL/6	CBA	2	4	83	83	83
2	C57BL/6	CBA	2	20	83	83	83
2b†	C57BL/6	CBA	2	20	100	100	100
3	C57BL/6	CBA	2	48	50	0	0
4	C57BL/6	CBA	2	72	0	—	—
5	—	CBA	0.5	4	100	83	83
6	C57BL/6	CBA	0.1	4	0	—	—
7	C57BL/6	AKR	2	4	100	100	83

*Spleen cells (5 × 10^7) and bone marrow cells (2 × 10^7).

†Groups of six mice were irradiated with 8.5 Gy 24 hours before transplantation.

‡Hours after irradiation that 0.25 mL of antibody was administered

with RmT_1 (data not shown). Table 4, tests 1, 2, and 7 shows that combined conditioning with radiation and RmT_1—injected 4 or 20 hours later—allowed a reduction in radiation to 5 or 6 Gy, a dose after which (in the absence of RmT_1) CBA mice were not chimeric (tested 3 weeks posttransplantation) when transfused with marrow from (C57BL/6xCBA) F_1 donors. Injection of RmT_1 1 day prior to irradiation with 6 Gy induced chimerism in only two of six mice (Table 4, test 6). Whereas 5 Gy followed by RmT_1 still induced chimerism, with 100% lymphocytotoxicity by CBA-anti-C57BL/6 immunoserum and 5% to 10% by C57BL/6-anti-CBA-serum, our results were the opposite after 4 Gy + RmT_1. Clear partial chimerism was not observed.

Immunocompetence of chimeras. Transplantation between homozygous mice mismatched in class I and class II MHC antigens raised the question of whether lymphohematopoetic reconstitution is satisfactorily achieved. A comparable gain in weight was observed in chimeras and control mice grafted with syngeneic and semiallogeneic marrow. Reconstitution of cellular immunity demonstrated tolerance of skin from marrow donor strain (survival >50 days) that was grafted on nine 60-day chimeras conditioned with 8.5 or 6 Gy

Table 4. Effect of Radiation Dose and Timing of Antibody Injection on Chimerism in Marrow Recipients Conditioned With RmT1 Rat IgG2b Anti-Thy-1

Test No	Irradiation* Dose (Gy)	Antibody Injection (h)†	Chimerism‡		
			Day 14	Day 21	Day 60
1	8.5	20	4/4	4/4	4/4
2	6	20	3/3	2/2	4/4
3	5	20	2/3	4/4	—
4	4	20	0/3	0/3	—
5	6	96	0/4	—	—
6	6	48§	2/6	2/6	—
7	6	4	4/4	2/2	2/2

*Twenty-four hours before transplantation of 5 × 10^7 spleen and 2 × 10^7 bone marrow C57BL/6 cells into CBA mice.

†Before transplantation.

‡Measured by microlymphocytotoxicity of lymph node cells with polyclonal CBA anti-C57BL/6 and C57BL/6 anti-CBA immune sera; number of mice with full chimerism *v* number of mice tested: >90% cells positive with CBA anti-C57BL/6 and <10% with C57BL/6 anti-CBA immune serum were typed as full chimeras. A reverse proportion was found in nonchimeric mice and in reversals.

§Test no. 2 represents 20 hours.

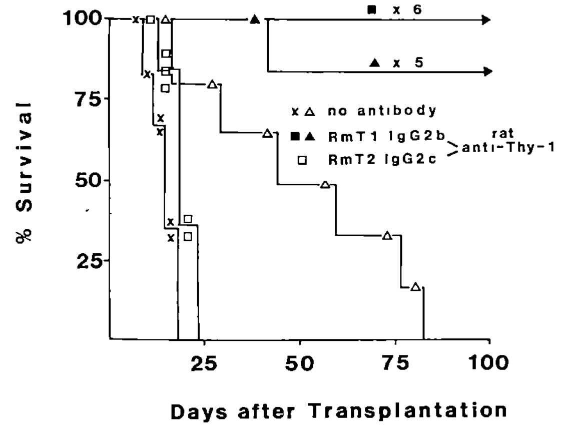

Fig 3. Survival of CBA mice after transplantation of spleen together with bone marrow cells (x,□,■) or of bone marrow cells alone (△,▲). CBA mice were conditioned with 8.5 Gy and 0.25 mL of rat anti-Thy-1 antibody (■,▲,□) or with irradiation alone (x,△).

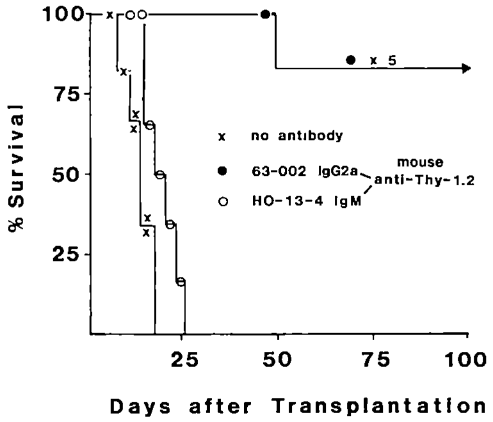

Fig 4. Survival of CBA mice after transplantation of spleen together with bone marrow cells from C57BL/6 donors. CBA mice were conditioned with 8.5 Gy and 0.25 mL of mouse anti-Thy-1.2 antibody.

and RmT_1. Skin from third-party strains (Balb/c) was rejected within 18 days. Primary humoral immune response to SRBCs was almost negative in 60-day chimeras conditioned with 8.5 or 6 Gy and RmT_1 (Table 5). After secondary immunization, however, antibody titers and plaque-forming cells rose to levels observed in CBA mice grafted with semiallogeneic and syngeneic mice.

DISCUSSION

We tested modalities of an approach for the simultaneous suppression of graft-*v*-host and host-*v*-graft reactions. Some basic conclusions from differences in the behavior of monoclonal antibodies regarding binding of Clq as the prerequisite for complement activation allowed us to separate antibodies of comparable specificity into isotypes inhibiting or not inhibiting T cell-induced complications in our marrow transplantation model. They also helped us to explain why chimerism was achieved after total body irradiation doses that could be reduced to 5 Gy.

When comparing a number of rat anti-Thy-1 mAbs of IgM, IgG2a, b, or c isotype, we previously observed that only IgG2b mAbs delayed the rejection of H-2-incompatible skin allografts[11] and suppressed GVHD following in vitro treatment of donor marrow without added complement.[8,9] We have now shown also that a mouse IgG2a, but not a mouse IgM or a rat IgG2c anti-Thy-1.2 mAb, suppresses GVHD when incubated with donor marrow in the absence of complement. The mouse IgG2a anti-Thy-1.2 isotype had previously been found to induce opsonization by macrophages, suppress reactivity of spleen and lymph node cells to T cell mitogens, and abolish the antibody response to SRBCs in vivo.[18] All Thy-1 antibodies of our study—independent of their monomorphic or polymorphic specificity—cross-inhibited each other at the Thy-1 antigen when tested in a radioimmunoassay.[9] Thus, neither clear differences in specificity nor avidity could explain why RmT_1 rat IgG2b and 63-002 mouse IgG2a anti-Thy-1 appeared less complement-dependent than did RmT_2 rat IgG2c and mouse HO-13-4 IgM anti-Thy-1 in the pretreatment of donor marrow for suppression of GVHD. We therefore postulated that rat IgG2b (of which we now have three samples produced in different laboratories, including our own) and mouse IgG2a anti-Thy-1 require no C′ during the in vitro donor cell treatment because they coat the donor T cells in vitro and inhibit them after transfer by exploiting the marrow recipient's complement system. The assumption of a better complement-activating capacity of these two isotypes was supported by the observation that rat IgG2b[12] and (in the present study) also mouse IgG2a anti-Thy-1 mAbs show a high binding affinity for Clq that the rat IgM, IgG2a, IgG2c, and the mouse IgM and IgGl anti-Thy-1 isotypes lack. Without heterologous complement, the latter, Clq-low-affine anti-Thy-1 antibody isotopes, had not suppressed GVHD in the present, fully mismatched animal model.[1,9] In all, we now have knowledge of seven anti-Thy-1 rat or mouse isotypes where affinity for Clq correlates with the immunosuppressive efficacy of antibodies. High affinity for Clq had originally been observed by Hughes-Jones and colleagues[24] in rat IgG2b antibodies against rat MHC alloantigens. Differences in Clq-binding affinity could best be revealed as a retention of Clq or the lack thereof after the antibody-coated T cells had undergone a wash-dilution step in the radioimmunoassay. High affinity for Clq requires in addition a proximity of antibodies and consequently of antigens on the cell membrane so that the multiheaded Clq can span a distance of ~30 nm between the C γ regions of at least two antibodies.[25]

Table 5. Immune Response to Sheep RBCs in Chimeric CBA Mice Conditioned With Irradiation and RmT_1

Test No.	Donor	Plaques per 10^6 Spleen Cells	Agglutination Log_2 Titer
		Primary Immunization	
1*	C57BL/6	13 ± 4	0
2	C57BL/6	6 ± 2	2
3	(C57BL/6 × CBA)F_1	261 ± 120	8
4	(C57BL/6 × CBA)F_1	745 ± 114	7
5	CBA	707 ± 207	8
		Secondary Immunization	
6*	C57BL/6	520/590† ±40 ±140	9
7	C57BL/6	355/587 ±66 ±30	9
8	(C57BL/6 × CBA)F_1	158/565 ±70 ±115	9
9	(C57BL/6 × CBA)F_1	130/160 ±8 ±16	9
10	CBA	120/294 ±51 ±143	9

Sixty days posttransplantation. Results are expressed as arithmetic means ± SD from groups of four mice

*Results of tests no 1 and 6 are from chimeras conditioned with 8.5 Gy All other chimeras had been conditioned with 6 Gy

†Direct/indirect plaques

The poor immunosuppression observed with HO-13-4 mouse IgM anti-Thy-1, with a cytotoxic titer of ~1:300,000, may appear surprising. Our transplantation model develops a mortality that is very difficult to inhibit. Semiallogeneic H-2 or certain minor-antigens-incompatible donor-recipient combinations have been more sensitive to IgM anti-Thy-1 antibodies.[9,26] The failure of the IgM antibody to bind Clq under the conditions of our RIA, and the high uptake of Clq by 63-002 mouse IgG2a anti-Thy-1.2 with its lower cytotoxic titer, shows how much better Clq-uptake correlates with in vivo immunosuppression by mAbs. Previous reports demonstrated that antibody-dependent cell-mediated cytotoxicity (ADCC) is promoted by various rat IgG2b[27] and by mouse IgG2a anti-Thy-1 mAbs.[28] It remains to be determined whether ADCC and Clq-binding affinity are interrelated, for instance, whether Fc binding to effector cells includes a link through Clq and/or further C′ components. Clq has indeed been reported to be expressed on bone marrow and peritoneal macrophages with an affinity for Fc portions of antigen-bound antibodies.[29]

Whatever the underlying mechanism for the remarkable immunosuppression of the two anti-Thy-1 isotypes may be in vivo, the practical implications are obvious: first, the capacity to fix complement in vitro, even at high antibody titers, is not a sufficient criterion for a T cell antibody to suppress secondary disease optimally, and second, there are isotypes of C′-fixing pan-T cell antibodies that are sufficient to allow preparation for transplantation of mismatched marrow even at sublethal conditioning doses.

Further modalities of the approach may have contributed to the prevention of acute and chronic GVHD in our animal model, for instance, the observed circulation time of the injected antibody activity, which lasted several days. A prolonged inhibitory effect on T cells may thus also have reached donor cells that escaped a 1-hour pretreatment in vitro. Our results show that the timing of the antibody injection is important. A delay of >24 hours after transplantation reversed immunosuppression. The effect of sublethal conditioning with Clq-affine anti-Thy-1 mAbs on colony-forming stem cells is under investigation. The transplantations were performed mainly with excess numbers of bone marrow and spleen cells and recipient mice <25 g to increase the sensitivity with regard to acute and chronic mortality from GVHD. We also found that a tenth (ie, 2×10^6 bone marrow and 5×10^6 spleen cells) of the usual cell amount can produce chimerism despite possible partial cross-reactions that have been reported for anti-Thy-1 mAbs and colony-forming hemopoietic stem cells.[30]

The very low primary immune response to SRBCs of our fully mismatched chimeras agrees with the concept of elements of restriction imposed by the H-2 type of thymus, in which immature T cells differentiate (reviewed in ref. 31). H-2 compatibility of lymphoid stem cells and thymus on both the I-A and K (or D) region is believed to be essential for the phenotypic manifestation of virus-specific, H-2–restricted cytotoxic T lymphocytes. This conclusion is in part based on reports[32,33] describing how, in a primary sensitization protocol, T cells from fully allogeneic chimeras fail to mount virus-specific H-2–restricted CTL responses. Using thymectomized F_1-radiation chimeras produced from T cell-depleted bone marrow and fully mismatched thymus grafts, however, we had already noticed a clear secondary immune response to SRBCs and, in vitro, the generation of alloreactive cytotoxic T lymphocytes.[34] This agrees with the good secondary immune response to SRBCs in our present chimeras. The rejection of third-party skin grafts indicated that the cellular immune response can also be reconstituted despite a mismatched radioresistant thymus epithelium.

In patients, transplantation of HL-A–mismatched bone marrow often fails even before GVHD develops Conventional high-dose total body irradiation which suppresses host-*v*-graft reactivity against minor antigens of HL-A–identical allografts frequently does not prevent rejection of HL-A–mismatched stem cells, often transfused in suboptimal numbers Therefore, more complete suppression of host-*v*-graft reactivity is a prerequisite for overcoming GVHD across HL-A barriers Side effects, however, limit present attempts to increase radiation doses. Sublethal conditioning with Clq-affine T cell antibodies should condition for bone marrow mismatched for both minor and major antigens. Finally, leukemic patients may also be considered for the described sublethal conditioning. The decreased antileukemic effect of the reduced radiation dose may be well compensated for by the early injection of antibodies that contribute to the cleansing of the leukemic patient from host cells. The remarkable antileukemic effect of the mouse IgG2a anti-Thy-1.2 isotype[28] is consistent with our preliminary experience with use of the rat IgG2b anti-Thy-1 isotype against a murine thymoma.

REFERENCES

1. Thierfelder S, Hoffmann-Fezer G, Rodt H, Doxiadis I, Eulitz M: Antilymphocytic antibodies and marrow transplantation. VI. Absence of immunosuppression in vivo after injection of monoclonal antibodies blocking graft-versus-host reactions and humoral antibody formation in vitro. Transplantation 35:249, 1983

2 Vallera DA, Soderling CCB, Carlson GJ, Kersey JH. Bone marrow transplantation across major histocompatibility barriers in mice Effect of elimination of T cells from donor grafts by treatment with monoclonal Thy-1.2 plus complement or antibody alone. Transplantation 31·218, 1981

3. Thierfelder S, Hoffmann-Fezer G, Rodt H, Kummer U: Cell manipulation with antibodies: Differential effects of anti-T and ATG, in Baum S, Ledney GD (eds): Experimental Hematology Today, Basel, Karger Verlag, 1982, p 103

4 Rodt HV, Thierfelder S, Eulitz M: Suppression of acute secondary disease by heterologous anti-brain serum. Blut 25:385, 1972

5. Rodt H, Thierfelder S, Eulitz M Anti-lymphocytic antibodies and marrow transplantation. III. Effect of heterologous anti-brain antibodies on acute secondary disease in mice. Eur J Immunol 4.25, 1974

6 Kolb HJ, Rieder I, Rodt H, Netzel B, Grosse-Wilde H, Scholz S, Schäffer E, Kolb E, Thierfelder S: Anti-lymphocytic antibodies and marrow transplantation VI Graft-versus-host tolerance in DLA-incompatible dogs following 'in vitro' treatment of bone marrow with absorbed antithymocyte globulin. Transplantation 27:242, 1979

7. Martin PJ, Hansen JA, Thomas ED: Preincubation of donor bone marrow cells with a combination of murine monoclonal anti-T-cell antibodies without complement does not prevent graft-versus-host disease after allogeneic marrow transplantation. J Clin Immunol 4.18, 1984

8. Thierfelder S, Hoffmann-Fezer G, Rodt H, Cobbold SP, Waldmann H: Influence of isotype on suppression of GVH with anti-Thy-1. Exp Hematol 12:373, 1984 (abstr)

9. Thierfelder S, Cobbold SP, Kummer U, Waldmann H, Schuh R: Antilymphocytic antibodies and marrow transplantation. VII Two out of nine monoclonal anti-Thy-1 antibodies used for pretreatment of donor marrow suppressed graft-versus-host reactions without added complement Exp Hematol 13:948, 1985

10. Ledbetter JA, Rouse RV, Micklem HS, Herzenberg LA. T cell subsets defined by expression of Lyt 1, 2, 3 and Thy-1 antigens. Two parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. J Exp Med 152:280, 1980

11. Cobbold SP, Thierfelder S, Waldmann H· Immunosuppression with monoclonal antibodies. A model to determine the rules for effective serotherapy. Mol Biol Med 1:285, 1983

12. Kummer U, Thierfelder S, Schuh R: Not only complement fixation but also complement affinity of monoclonal anti-T greatly affect the suppression of GvHD. Exp Hematol 13.352, 1985 (abstr)

13. Mittal KK, Mickey MR, Singal DP, Terasaki PI· Serotyping for homotransplantation. XVIII. Refinement of microdroplet lymphocyte cytotoxicity. Transplantation 6·913, 1968

14. Jerne D, Nordin AA: Plaque formation in agar by single antibody-producing cells Science 140:405, 1963

15. Dresser DW, Wortis HH: Use of an antiglobulin serum to detect cells producing antibody with low haemolytic efficiency. Nature 208.859, 1965

16. Bazin H: Production of rat monoclonal antibodies with the Lou rat non-secreting IR983F myeloma cell line. Prot Biol Fluids 29.615, 1982

17 Marshak-Rotstein A, Fink P, Gridley T, Raulet DH, Bevan MJ, Gefter ML: Properties and application on monoclonal antibodies directed against determinants of the Thy-1 locus. J Immunol 122.2491, 1979

18. Opitz HG, Opitz U, Hewlett G, Schlumberger HD A new model for investigations of T-cell functions in mice: Differential immunosuppressive effects of two monoclonal anti-Thy-1.2 antibodies. Immunobiology 160·438, 1982

19. Rodt H, Thierfelder S, Thiel E, Götze D, Netzel B, Huhn D, Eulitz M: Identification and quantitation of human T-cell antigen by antisera purified from antibodies crossreacting with hemopoietic progenitors and other blood cells Immunogenetics 2.411, 1975

20. Cooper NR: The clinical complement pathway: Activation and regulation on the first complement component, in Dixon FJ (ed): Advances in Immunology, vol 37. Orlando, Fla, Academic Press, 1985, p 151

21 Zubler RH, Lange G, Lambert PH, Miescher PA: Detection of immune complexes in unheated sera by a modified ^{125}I-Clq binding test. Effect of heating on the binding of Clq by immune complexes and application of the test to systemic lupus erythematosus J Immunol 116:232, 1976

22. Kummer U, Thiel E, Doxiadis I, Eulitz M, Sladoljev S, Thierfelder S. Tritium radiolabeling of antibodies to high specific activity with *N*-succinimidyl (2,3-^{3}H) propionate Use in detecting monoclonal antibodies. J Immunol Methods 42·367, 1981

23. Reif AE, Allen JM: Mouse thymic Iso-antigens. Nature 209:521, 1966

24. Hughes-Jones NC, Gorick BD, Howard JC: The mechanism of synergistic complement mediated lysis of rat red cells by monoclonal IgG antibodies. Eur J Immunol 13:635, 1983

25. Knobel HR, Villiger W, Isliker H. Chemical analysis and electron microscopy studies of human Clq prepared by different methods. Eur J Immunol 5 78, 1975

26. Hamilton BL, Harris D: Prevention of graft-versus-host disease using antibody to Thy-1. A role for complement in vivo Transplantation 40:90, 1985

27. Hale G, Clark M, Waldmann H. Therapeutic potential of rat monoclonal antibodies: Isotype specificity of antibody-dependent cell-mediated cytotoxicity with human lymphocytes. J Immunol 134:3056, 1985

28. Denkers EY, Badger CC. Ledbetter JA, Bernstein ID: Influence of antibody isotype on passive serotherapy of lymphoma. J Immunol 135:2183, 1985

29. Heinz HP, Dlugonska H, Rüde E, Loos H. Monoclonal anti-mouse macrophage antibodies recognize the globular proteins of Clq, a subsequent of the first component of complement. J Immunol 133:400, 1984

30. Williams DE, Boswell HS, Floyd AD, Broxmeyer HE. Pluripotential hematopoietic stem cells in post-5-fluorouracil murine bone marrow express the Thy-1 antigen. Immunology 135.1004, 1985

31. Möller, G (ed): MHC Restriction on Anti-Viral Immunity. Immunological Reviews, vol 58. Copenhagen, Munksgaard, 1981

32. Zinkernagel RM, Callahan GN, Althage A, Cooper S, Streilein JW, Klein J: The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: Evidence for T help. J Exp Med 147:897, 1978

33 Zinkernagel RM, Callahan GN, Althage A, Cooper J, Klein PA, Klein J: On the thymus in the differentiation of "H-2 self-recognition" by T cells: Evidence for dual recognition. J Exp Med 147:882, 1978

34. Wagner H, Röllinghoff M, Rodt H, Thierfelder S: T cell-mediated cytotoxic immune responsiveness of chimeric mice bearing a thymus graft fully allogenic to the graft of lymphoid stem cells. Eur J Immunol 10:521, 1980

Prostacyclin Expression by a Continuous Human Cell Line Derived From Vascular Endothelium

By Joseph E. Suggs, Michael C. Madden, Mitchell Friedman, and Cora-Jean S. Edgell

Prostacyclin is primarily an endothelial cell product. It contributes to the important role of endothelium in maintaining the fluidity of blood by inhibiting platelet aggregation and by promoting vasodilation. Endothelial cells in culture tend to senesce, and the level of prostacyclin expression decreases. A permanent human cell line, EA.hy 926, derived from a fusion of primary endothelial cells with cells of a less differentiated line, has been found to sustain basal and stimulated levels of prostacyclin synthesis.
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ARACHIDONIC ACID metabolites are autacoids that modulate cellular functions near the site at which they are produced.[1] These structurally related metabolites have a wide range of physiological effects, some of which oppose others. Two arachidonic acid metabolites affect platelet aggregation at a site of vascular injury. Aggregation is promoted by platelet thromboxane A_2,[2] but it is limited by prostacyclin produced by endothelial cells.[3,4] Thrombin induces prostacyclin production by endothelial cells,[5] and these cells can also convert precursors released by aggregated platelets to prostacyclin.[6] Thrombosis may thus be limited by prostacyclin from downstream endothelial cells. Prostacyclin is the major prostaglandin produced by endothelial cells; relatively, they produce much more of this eicosanoid than do other cell types.[7–9] The cell type-specific synthesis and the short half-life of prostacyclin[10] contribute to the locational control of its effects.

In addition to inhibiting platelet aggregation, prostacyclin acts as a potent vasodilator by causing a relaxation response in vascular smooth muscle.[11,12] Agents such as angiotensin II, bradykinin, histamine, and thrombin can stimulate endothelial cells to produce prostacyclin.[13–15] Thus, the endothelial cell lining of the vascular system facilitates blood flow by prostacyclin-mediated vasodilation as well as by prevention of excessive platelet aggregation.

The study of prostacyclin synthesis and regulation in isolated endothelial cells is hindered by the limited replication potential of these cells and their tendency to senesce in culture with the production of prostacyclin deteriorating in subcultures[16,17] Therefore, the potential for sustained expression of prostacyclin by a permanent cell line derived from human umbilical vein endothelial cells (HUV-EC) was investigated. This cell line, EA.hy 926,[18] is a hybrid resulting from fusion of primary endothelial cells with cells selected from the continuous human line A549.[19] The EA.hy 926 line represents cells that were clonally isolated following hybridization and subcloned again at passage 35. Since subcloning, the cells have been passaged at least 30 times more. The cells sustain expression of many differentiated functions of endothelium after exceeding 200 cumulative population doublings in culture. These functions include von Willebrand factor,[18,20] thrombomodulin,[21] and tissue plasminogen activator.[22] Data presented here demonstrate that this cell line also produces basal levels of prostacyclin and responds to physiological stimulation to produce higher levels.

METHODS

Cell culture. EA.hy 926 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin at 100 U/mL, streptomycin at 100 µg/mL, and in the presence of HAT (100 µmol/L of hypoxanthine, 0.4 µmol/L of aminopterin, 16 µmol/L of thymidine). These cells were generally subcultured weekly at a relative cell density of 1.20 The EA.hy 926 cells used in these studies had been passed more than 60 times.

A549/8, one of the progenitor strains of the EA hy 926 line, is a thioguanine-resistant strain[23] selected from the human lung adenocarcinoma line established by Lieber and co-workers.[19] It was grown in RPMI 1640 supplemented with 7% Nu-Serum (Collaborative Research, Bedford, Mass), penicillin at 100 U/mL, streptomycin at 100 µg/mL, and in the presence of 0.1 µmol/L of thioguanine.

HUV-EC were isolated as described by Gimbrone[24] and grown in RPMI 1640 supplemented with 15% fetal bovine serum, bovine hypothalamus extract,[25] penicillin at 100 U/mL and streptomycin at 10 µg/mL Unpassaged cultures of HUV-EC were used in these studies for comparison with the EA.hy 926 cell line.

Sample preparation and radioimmunoassay Confluent cell layers in multiwell plates were rinsed twice with unsupplemented RPMI 1640 medium 48 to 72 hours after the previous change of serum-containing culture medium and were then incubated in RPMI 1640 at 0.2 mL/cm^2 After 1 hour, the medium samples were collected from each culture to represent its basal level of prostacyclin production. Bovine thrombin (Parke-Davis, Morris Plains, NJ, NDC 0071-1356-01) was then added to each culture at 0.5 U/mL in RPMI 1640, and samples were collected after a 5-minute incubation to represent the level of prostaglandins released by stimulated cells All samples were centrifuged to remove cellular debris, and stored at −70 °C for immunological analysis

A radioimmunoassay (RIA) method (New England Nuclear, Boston) was used to assess levels of prostacyclin in these samples as 6-keto-PGF$_{1\alpha}$, the stable derivative. The RIA uses ^{3}H-labeled 6-keto-PGF$_{1\alpha}$ and was capable of distinguishing between standards of 5 pg/100 mL and 10 pg/100 mL. After the culture supernatant samples were removed, the protein contents of cell layers were determined so that results from cell lines that grow to different cell densities could be normalized in terms of protein mass. Trypsinized cells were lysed in water, and the protein content was measured by the Bradford assay.[26]

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Table 1. Prostacyclin Production by Three Cell Types

Cell Line	Basal	Stimulated	Ratio
	(ng 6 keto-PGF$_{1\alpha}$ per mg protein)		Stimulated/Basal
HUV-EC (n = 4)	9.25 ± 2.01	1,642 ± 330	177
EA.hy 926 (n = 6)	3.99 ± 1.28	251 ± 63	63
A549/8 (n = 6)	0.53 ± 0.13	3 ± 1	6

These radioimmunoassay (RIA) data represent the amounts of prostacyclin produced in 1 hour under basal conditions divided by 12 (or amounts per 5 minutes) and the amounts produced within 5 minutes after adding thrombin. The mean level ± the SD for a number (n) of parallel cultures is presented for each cell type. The human umbilical cord endothelial cells (HUV-EC) were primary cultures derived from a single umbilical cord; the EA.hy 926 cultures were from passage 67.

High-performance liquid chromatography (HPLC) analysis. To radiolabel arachidonic acid metabolites for detection following HPLC fractionation, rinsed 25-cm^2 monolayers were incubated in the presence of 5 μCi of ^{3}H-arachidonic acid at 50 Ci/mmol (New England Nuclear) in 5 mL of RPMI 1640. After 19 hours, the exogenous arachidonic acid solution was removed, the cultures were rinsed twice, and fresh RPMI 1640 was added. This medium was collected after 1 hour to represent the basal production of arachidonic acid metabolites. It was replaced with 5 mL of RPMI 1640 containing 0.5 U/mL of thrombin, which was collected after 5 minutes to represent the arachidonic acid metabolites produced by stimulated cells.

For HPLC fractionation, the arachidonic acid metabolites were extracted from RPMI 1640 into organic solvents. The RPMI 1640 samples were first adjusted to pH 3.5 with 9% formic acid, then extracted with 2 vol of cyclohexane/ethyl acetate (1:1), followed by 2 vol of ethyl acetate. The combine extracts of each sample were evaporated in a stream of nitrogen at 45 °C, and the residue was dissolved in methanol 200 μL/25 cm^2 of confluent cells. Recovery of radiolabeled 6-keto-PGF$_{1\alpha}$ through this extraction procedure was 94%.

Arachidonic acid metabolites were fractionated by the HPLC method of VanRollins and colleagues[27] using a column of Altex 5-μm Ultrasphere ODS particles and a gradient of acetonitrile in aqueous phosphoric acid pH 2 for elution. Each injected sample was 150 μL of the extract dissolved in methanol. Fractions containing ^{3}H-labeled metabolites were detected by scintillation counting. The components were identified by comparing their elution volumes with those of purified standards chromatographed separately and detected by ultraviolet (UV) abosrbance.

RESULTS

EA.hy 926 cells were found to produce relatively high levels of prostacyclin as determined by the RIA data shown in Table 1. The results per mass of cell protein are presented for the purpose of comparing the hybrid cell line with its two progenitor cell types, all of which have different cell sizes and culture densities at confluence. Under basal conditions, the hybrid cells produced much more prostacyclin than A549/8 cells, although less than primary cultures of HUV-EC. The EA.hy 926 cells also responded strongly to thrombin stimulation to produce 63-fold more prostacyclin. The response of HUV-EC was even stronger than that of the hybrid cells, but the response of A549/8 cells was much weaker.

The HPLC method also demonstrated that the 6-keto-PGF$_{1\alpha}$ prostacyclin derivative was a product of EA.hy 926 cells, as shown in Fig 1. The amount produced under basal conditions was substantially increased by thrombin stimulation. Low levels of certain other products of the cyclooxygenase pathway were also detectable in samples from EA.hy 926 cells and from HUV-EC cells, but the stable prostacyclin derivative was the major component. As expected, primary cultures of HUV-EC produced even more prostacyclin, but even with stimulation A549/8 cells did not produce levels detectable by this method.

Prostacyclin release by EA.hy 926 cells could be stimulated by melittin and by arachidonic acid as well as by thrombin. When cells that had been stimulated with 20 mmol/L of arachidonic acid were washed and then restimulated within 15 minutes, the amount of prostacyclin released was only 55% of that released at the initial stimulation. Dejana and colleagues[28] and others have shown that cells are refractory to restimulation for several hours and have suggested that cyclooxygenase may be irreversibly inactivated by arachidonic acid metabolism.

Table 2. Prostacyclin Production by Different Cell Types in Culture

Cell Type	6-keto-PGF$_{1\alpha}$	Species	Anatomic Source	Reference
Endothelium	1.47ng/10^6 cells/h	Cow	Pulmonary artery	30
Endothelium	20 ng/10^6 cells/h*	Cow	Pulmonary artery	31
Smooth muscle	0.175 ng/10^6 cells/h*	Rabbit	Pulmonary artery	
Fibroblastic	0.085 ng/10^6 cells/h*	Human	Embryonic lung	
Epithelium	0.175 ng/10^6 cells/h*	Rat	Prostate	
Endothelium	8.8 ng/10^6 cells/h	Human	Umbilical vein	6
Endothelium	14.4 ng/10^6 cells/h	Human	Umbilical vein	32
	29.2 ng/10^6 cells/h		Pulmonary vein	
	21.6 ng/10^6 cells/h		Pulmonary artery	
Endothelium	27 ng/10^6 cells/h	Pig	Aorta	33
Endothelium	8.4 ng/10^6 cells/h	Cow	Aorta	34
Smooth muscle	0.5 ng/10^6 cells/h	Rabbit	Aorta	35
Macrophage	0.035 ng/10^6 cells/h	Mouse	Peritoneum	

Published values for the amount of prostacyclin derivative measured in medium from several cell types isolated from various species.

*Data from this reference were converted to comparable units by assuming the use of 5 mL of medium per 20-cm^2 culture dish.

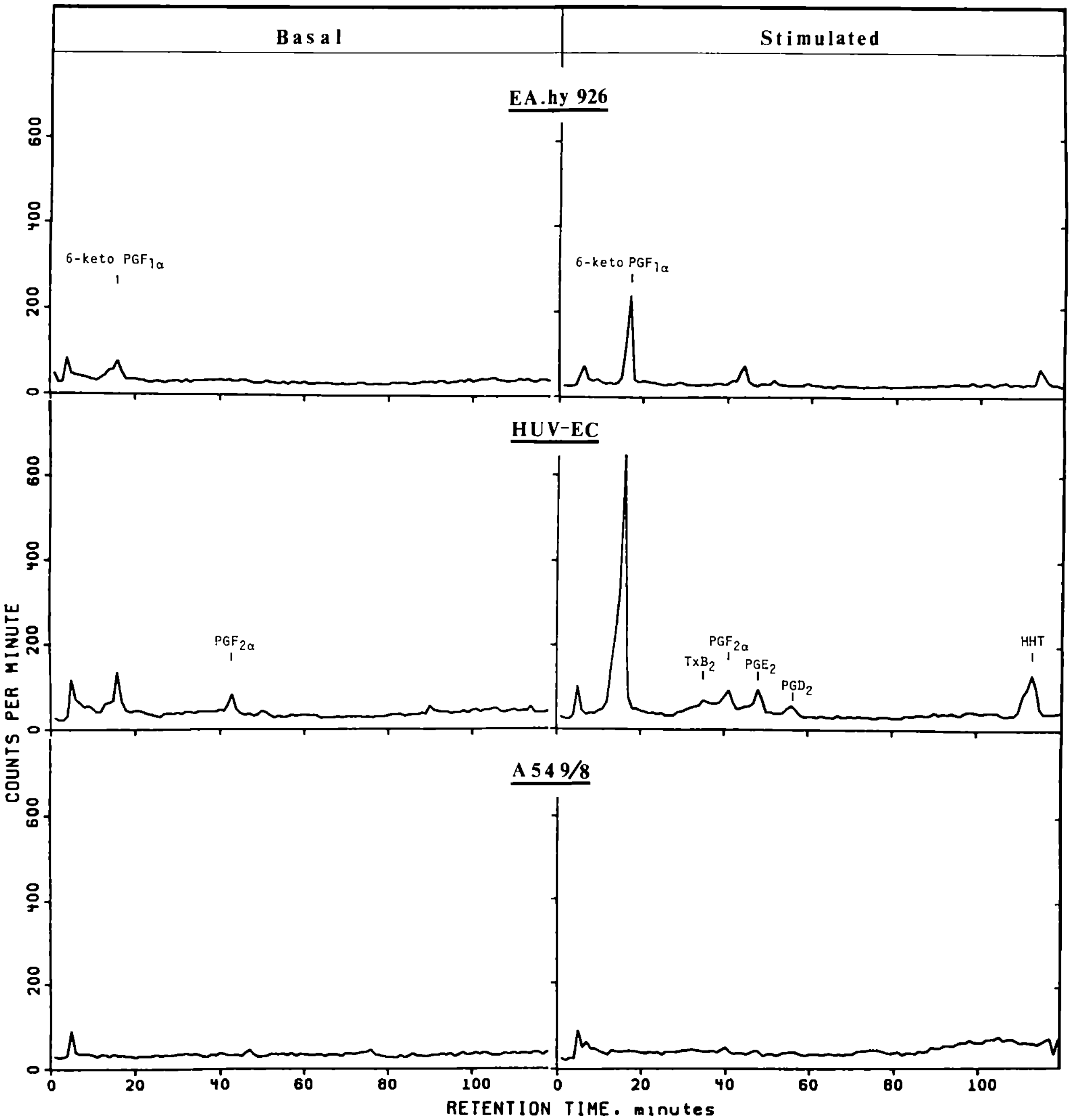

Fig 1. Chromatographic identification of arachidonic acid metabolites generated by three different cell types under basal and thrombin-stimulated conditions. Culture supernatants were extracted and analyzed by high-pressure liquid chromatography (HPLC) following incorporation of ^{3}H-arachidonic acid by confluent cultures of each cell line. The elution positions of purified standards are indicated.

DISCUSSION

It is clear that the premanent EA.hy 926 cells have retained the capacity to produce prostacyclin in a range close to that produced by HUV-EC in primary cultures. This trait is presumably inherited from the endothelial cell progenitor of the hybrid cell line; thus, prostacyclin expression has been sustained in the EA.hy 926 line for hundreds of cell generations. Although the levels of prostacyclin fluctuated when measured on different occasions, no consistent trend was detectable in the results of various experiments performed with the hybrid line over a 6-month interval. The amount of prostacyclin released by HUV-EC also varied from one primary culture to another, and other researchers have noted variability among parallel cultures of the same HUV-EC preparation.[17] The EAhy 926 cells are like HUV-EC in this respect. The variability may reflect conditions that are not well controlled by conventional cell culture methods. In contrast to EA.hy 926 cells and HUV-EC, the A549/8 cells produced relatively very little prostacyclin under basal or stimulated conditions, as expected for most nonendothelial cell types. Data from several investigators have indicated that substantial levels of prostacyclin production is a relatively specialized property of endothelial cells, although there is variability in the results from different laboratories and from different species. Some of these data are summarized in Table 2. In comparable units, the EA.hy 926 cells produce the prostacyclin derivative at a basal level of 1 to 4 ng/10^6 cells per hour.

In addition to prostacyclin, endothelial cells produce other arachidonic acid metabolites, the spectrum of which is relatively specific for this cell type. Some of these metabolites can be identified in the HPLC analyses, as indicated in Fig 1. $PGF_{2\alpha}$ and HHT are common to stimulated EA.hy 926 cells and HUV-EC, but not detectable in medium from nonendothelial cell type A549/8. Additional similarities were observed between primary HUV-EC and EA.hy 926 cells in contrast to A549/8 cells in the regions of the chromatograms where the hydroxyeicosatetraenoic acids (HETEs) elute (data not shown).

The vigorous growth, unlimited replication potential, and clonal purity of the human endothelial cell-derived EA.hy 926 line could facilitate studies of the pathways and regulation of prostacyclin biosynthesis.

REFERENCES

1 Marcus AJ, Broekman MJ, Safier LB, Ullman HL, Weksler BB, Jaffe EA, Kaplan KL, Wong KTH: Essential fatty acids in platelets and endothelial cells, in Nossel HL, Vogel HJ (eds)· Pathobiology of the Endothelial Cell. Proceedings of the Sixth P & S Biomedical Sciences Symposia. Orlando, FL, Academic Press, 1982, p 311

2. Hamberg M, Svenson J, Samuelsson B. Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci USA 72:2994, 1975

3 Moncada S, Gryglewski RJ, Bunting S, Vane JR: An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 263·663, 1976

4. Johnson RA, Morton DR, Kinner JH, Gorman RR, McGuire JC, Sun FF: The chemical structure of prostaglandin X (prostacyclin). Prostaglandins 12·915, 1976

5. Weksler BB, Ley CW, Jaffe EA Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore A23187. J Clin Invest 62:923, 1978

6. Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ: Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J Clin Invest 66:979, 1980

7. Moncada S, Herman AG, Higgs EA, Vane JR: Differential formation of prostacyclin (PGX or PGI_2 by layers of the arterial wall. An explanation for the antithrombotic properties of vascular endothelium Thromb Res 11:323, 1977

8. Weksler BB, Marcus AJ, Jaffe EA: Synthesis of prostaglandin I_2 (prostacyclin) by cultured human and bovine endothelial cells Proc Natl Acad Sci USA 74.3922, 1977

9. Baenziger NL, Bercherer PR, Majerus PW· Characterization of prostacyclin synthesis in cultured human arterial smooth muscle cells, venous endothelial cells and skin fibroblasts. Cell 16:967, 1979

10. Pace-Asiak C, Wolfe LS. A novel prostaglandin derivative formed from arachidonic acid by rat stomach homogenates Biochemistry 10.3657, 1971

11. Bunting S, Gryglewski RJ, Moncada S, Vane JR: Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation. Prostaglandins 12.897, 1976

12. O'Grady J, Warrington S, Moti MJ, Bunting S, Flower R, Fowle ASE, Higgs EA, Moncada S: Effects of intravenous infusion of prostacyclin (PGI_2) in man Prostaglandins 19:319, 1980

13. Lonchampt MD, Romquin N, Bonne C, Regnault F. The effect of angiotensin II on PGI_2 production by endothelial cells in culture. Thromb Haemost 46·39, 1981 (abstr)

14. Hong SL. Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein Thromb Res 18:787, 1980

15. Baenziger NL, Force LE, Becherer PR: Histamine stimulates prostacyclin synthesis in cultured human umbilical vein cells. Biochem Biophys Res Commun 92:1435, 1980

16. Ager A, Gordon JL, Moncada S, Pearson JD, Salmon JA, Trevethick MA Effects of isolation and culture on prostaglandin synthesis by porcine aortic endothelial and smooth muscle cells J Cell Physiol 110:9, 1982

17. Levin RI, Weksler BB, Marcus AJ, Jaffe EA: Prostacyclin production by endothelial cells, in Jaffe EA (ed). Biology of Endothelial Cells. The Hague, Martinus Nijhoff, 1984

18. Edgell CJS, McDonald CC, Graham JB: Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 80:3734, 1983

19. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro GJ A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 17 62, 1976

20. van Oost BA, Edgell CJS, Hay CW, MacGillivray RTA. Isolation of a human von Willebrand Factor cDNA from the hybrid endothelial cell line EA.hy 926. Can J Biochem (in press)

21 DeBault LE, Esmon NL, Esmon CT, Edgell CJS. Thrombomodulin expressed in human endothelial cell hybrid Fed Proc 43·2912a, 1984 (abstr)

22. Emeis JJ, Rijken DC, Edgell CJS Fibrinolytic characteristics of a human endothelial hybrid cell line Thromb Haemost 54:193, 1985 (abstr)

23 Edgell CJS, Gazdar AF, Minna JD· Expression of feline xenotropic RNA tumor virus in hybrids between permissive human and non-permissive mouse cells Int J Cancer 24 826, 1979

24. Gimbrone MA Jr: Culture of vascular endothelium Prog Hemost Thromb 3:1, 1976

25 Maciag TJ, Cerundolo J, Ilsley S, Kelley PR, Forand R: An endothelial cell growth factor from bovine hypothalamus: Identification and partial purification Proc Natl Acad Sci USA 76·5674, 1979

26. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976

27 VanRollins M, Ho SHK, Greenwald JE, Alexander M, Dorman NJ, Wong LK, Horrocks LA: Complete separation by high performance liquid chromatography of metabolites of arachidonic acid from incubation with human and rabbit platelets. Prostaglandins 20.571, 1980

28 Dejana E, Balconi G, De Castellarnau C, Barbieri M, Vergara-Dauden M, De Gaetano G: Prostacyclin production by human

endothelial and bovine smooth muscle cells in culture: Effect of repeated stimulation with arachidonic acid, thrombin, and ionophore A23187. Biochim Biophys Acta 750:261, 1983

29. Shier WT: Activation of high levels of endogenous phospholipase A$_2$ in cultured cells. Proc Natl Acad Sci USA 76:195, 1979

30. Friedman M, Madden MC, Saunders DS, Gammon K, Kwock LK Ozone inhibits prostacyclin synthesis in pulmonary endothelium. Prostaglandins 30·1069, 1985

31. Polgar P, Douglas WHJ, Terracio L, Taylor L: Release of arachidonic acid and its conversion to prostaglandins in variance diploid cell types in culture. Adv Prostaglandin Thromb Res 6:225, 1981

32 Johnson AR, Callahan KS, Tsai SC, Campbell WB: Prostacyclin and prostaglandin biosynthesis in human pulmonary endothelial cells Bull Eur Physiopathol Respir 17:79, 1981

33 Whorton AR, Young SL, Data JL, Barchowsky A, Kent RS: Mechanism of bradykinin-stimulated prostacyclin synthesis in porcine aortic endothelial cells Biochim Biophys Acta 712:79, 1982

34. Ingerman-Wojenski C, Silver MJ, Smith JB: Bovine endothelial cells in culture produce thromboxane as well as prostacyclin. J Clin Invest 67:1292, 1981

35. Pomerantz KB, Tall AR, Feinmark SJ, Cannon PJ: Stimulation of vascular smooth muscle cell prostacyclin and prostaglandin E$_2$ synthesis by plasma high and low density lipoproteins. Circ Res 54:554, 1984

Corticosteroids Reversibly Inhibit Chemotactic Peptide-Receptor Binding and Granulocyte Response, yet Allow Desensitization and Receptor Down-Regulation

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Inhibition of complement-mediated granulocyte aggregation has recently been proposed as a mechanism of action of high-dose corticosteroids in shock states. Such inhibition may be effected through alteration of receptor function. Methylprednisolone inhibits binding of the synthetic chemotaxin f-methionine-leucine-phenylalanine (FMLP) to its surface receptor on granulocytes in a dose-dependent manner by slowing the association rate of the FMLP-receptor interaction without altering receptor number. Because the half-life of high-dose methylprednisolone in vivo is short, we examined the reversibility of its effects on granulocyte aggregation and receptor function; both effects were readily reversed by washing. Furthermore, methylprednisolone, at concentrations that profoundly inhibited the granulocyte aggregation response to FMLP, allowed the occurrence of FMLP-receptor down-regulation and granulocyte desensitization to further stimulation by FMLP. We conclude that methylprednisolone at concentrations that inhibit granulocyte aggregation can slow the rate of the FMLP-receptor interaction while simultaneously allowing receptor down-regulation and granulocyte desensitization to proceed. Transient blockade of granulocyte function with concomitant desensitization and receptor down-regulation may be important in the clinical effects of very high-dose corticosteroids such as are administered in shock and could partially explain how drugs with such short half-lives administered intermittently could exert a beneficial effect in shock states. Similar observations may hold for other physiologic stimuli.
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RECENT EVIDENCE has suggested that complement-stimulated granulocytes are important in the pathogenesis of a variety of clinical disorders, including the adult respiratory distress syndrome,[1-4] the vasculitis of cholesterol embolization syndrome,[5] hemodialysis neutropenia,[6-9] and myocardial infarction.[10] High-dose corticosteroids inhibit granulocyte responses to activated complement in vitro and in vivo,[1,11,12] and this inhibition may explain in part the reported efficacy of high-dose corticosteroids in the adult respiratory distress syndrome, experimental endotoxic shock, and myocardial infarction.[1,13-22] High-dose corticosteroid therapy has been tried in other diseases as well, such as rheumatoid arthritis and idiopathic rapidly progressive glomerulonephritis, and further trials are being contemplated.[23-26] We have also shown that high-dose corticosteroids inhibit the binding of the synthetic chemotaxin *N*-formyl-met-leu-phe (FMLP) to its specific receptor on the granulocyte surface, and suggested that this inhibition may be a mechanism of the effect of high-dose corticosteroids on granulocyte responses to chemotactic stimuli.[27] Although its receptor is distinct from that of the chemotactic complement fragment, C5a,[28] FMLP mimics many of the effects of activated complement, including chemotaxis and aggregation of granulocytes, and in vivo induction of neutropenia.[27,29] Furthermore, FMLP-induced granulocyte aggregation is inhibited by the same concentrations of corticosteroids as inhibit C5a-induced aggregation.[1]

The plasma half-life of high-dose corticosteroids is quite short,[25,30,31] and other researchers have reported no alteration in granulocyte function following pulse methylprednisolone sodium succinate (MP) therapy in vivo.[32] We therefore tested the reversibility of the effects of MP on granulocyte aggregation and FMLP receptor function and found that corticosteroid-induced inhibition of receptor function and granulocyte aggregation could be reversed by washing. We next questioned whether methylprednisolone might be capable of altering the kinetics of the stimulus/receptor interaction, thereby inhibiting the granulocyte response to stimulation, yet allowing a slow increase in receptor occupancy with resulting down-regulation/desensitization of the cell to further stimulus following removal of the drug. We found that methylprednisolone was capable of simultaneously inhibiting granulocyte response to FMLP, while decreasing the rate of FMLP binding, yet allowing receptor down-regulation and granulocyte desensitization.

METHODS

Cell preparation Granulocytes were prepared from heparinized human venous blood by the previously described modification of the method of Boyum [6,33] Cells were suspended at appropriate concentrations in Hanks' balanced salt solution (HBSS), pH 7.4, (M. A Bioproducts, Bethesda, Md); HBSS containing 5 mg/mL of bovine serum albumin (Sigma, St. Louis) (HBSS–BSA); or in HBSS containing 5 mg/mL of human serum albumin (Cutter Biologicals, Berkeley, Calif) (HBSS–HSA). Differential cell counts performed on Wright's stained cells revealed greater than 95% polymorphonuclear cells (PMNs)

Preparation of solutions. Preservative and filler-free MP were kindly provided by the Upjohn Company (Kalamazoo, Mich) and dissolved in isotonic phosphate-buffered saline (PBS), pH 7 4

FMLP (Peninsula Laboratories, Inc, San Carlos, Calif) was dissolved in dimethylsulfoxide (DMSO) at 10 mmol/L, diluted in PBS to 1 mmol/L, and stored at −70 °C in aliquots until use

Zymosan-activated plasma (ZAP) was prepared as previously described[6] by incubating 2 mg of zymosan per milliliter of heparinized (1 U/mL) human plasma for 30 minutes at 37 °C, followed by removal of zymosan by centrifugation at 10,000 g for 30 minutes at 4 °C Aliquots were stored at −70 °C until use.

Disodium succinate (Sigma), and ^{3}H-FMLP (specific activity 56.9 Ci/mmol, New England Nuclear, Boston) were dissolved in PBS at appropriate concentrations

Kinetic analysis of the FMLP receptor interaction Although the relationships between the kinetics of interaction of ligands and

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cell-bound receptors as compared with the interaction of ligands and solubilized molecules may be quite complex, it is possible to obtain useful information using live cells.[34-36] Analysis of equilibrium binding of FMLP receptors was determined as described by Williams and colleagues[28] with minor modifications[27] Cells were suspended in phosphate-buffered saline (PBS) containing 0.5 mg/mL of BSA (PBS–BSA) at 5 to 10 × 10^7 cells/mL One hundred microliters of cell suspension was mixed with 17 μL of corticosteroid or PBS and incubated for 2 minutes at 22 °C, the cells were then cooled to 4 °C and added to 35 μL of various concentrations of ^{3}H-FMLP and 15 μL of either PBS or 10^{-4} mol/L of unlabeled FMLP in a 12 × 75-mm polypropylene tube and incubated at 37 °C or 4 °C as indicated for 20 minutes with occasional shaking. Each sample was then mixed with 3 mL of 0 °C PBS–BSA and filtered through a Whatman GF/C filter, and the retentate was washed with 12 mL of 0 °C PBS–BSA. Filters were then dried, added to 6 mL of Aquasol-2 (New England Nuclear) and counted in a Beckman scintillation counter Unless otherwise specified, all data are expressed as specific binding (binding of ^{3}H-FMLP in buffer minus binding of ^{3}H-FMLP in the presence of 10^{-5} mol/L of unlabeled FMLP). Nonspecific binding, defined as the amount of ^{3}H-FMLP binding observed in the presence of 10^{-5} mol/L of unlabeled FMLP, ranged from 5% to 18% of the total amount of ^{3}H-FMLP bound and was <10% of the total ^{3}H-FMLP binding in most experiments. Time course experiments revealed specific ^{3}H-FMLP binding to whole cells at 4 °C plateaued by 20 minutes. Equilibrium binding data were analyzed by the method of Scatchard[37] where $B/F = B_0K_{eq} - BK_{eq}$, and B = concentration of bound ^{3}H-FMLP, B_0 = total receptor concentration, and F = free ^{3}H-FMLP concentration.

Dissociation kinetics were determined as previously described.[27] Cells, (5 × 10^7 cells/mL) were incubated with receptor-saturating concentrations of ^{3}H-FMLP in HBSS at 4 °C for 20 minutes. A large excess of unlabeled FMLP or MP (final concentration 5 mg/mL) was then added in a small volume at t = 0, at various time intervals, aliquots were taken, filtered through a Whatman GF/C filter, washed with cold HBSS-BSA, and the filters were counted as described above Simultaneous controls were performed in the presence of excess unlabeled FMLP to measure nonspecific binding.[27] The resulting specific dpm were calculated and the concentration of cell-bound ^{3}H-FMLP was analyzed assuming the relationship:

$$RF \xrightarrow{k_r} R + F \quad \text{or} \quad d/dt\,[RF] = -k_r[RF],$$

where [RF] = the concentration of bound receptor-^{3}H-FMLP complex, [R] = the free receptor concentration, and [F] = the free ^{3}H-FMLP concentration.[27,38]

Association kinetics were determined as described.[27] Cells were incubated in HBSS in the presence or absence of 0.125 mg/mL of MP for 2 minutes at 22 °C; the cells were then cooled to 4 °C, and ^{3}H-FMLP was then added to the extent that the initial ^{3}H-FMLP concentration was at least 10 times the initial concentration of receptor sites (determined by equilibrium binding in the presence of excess ^{3}H-FMLP.) At various time intervals, samples were removed, filtered, washed, and counted as described above Although simultaneous experiments were performed in the presence of excess unlabeled FMLP to determine nonspecific binding, this correction was insignificant in most association experiments. The resulting dpm bound were calculated, and the concentrations of bound and free ^{3}H-FMLP were analyzed assuming the relationship.

$$R + F \underset{k_r}{\overset{k_f}{\rightleftharpoons}} RF \quad \text{or} \quad d/dt\,[RF] = k_f\,[R]\,[F] - k_r[RF],$$

where [R] = the free receptor concentration and [F] = the free ^{3}H-FMLP concentration. Because $k_r \ll k_f$ in this system, we may ignore the dissociation reaction during the brief time course of these experiments. To confirm that the reaction followed second-order kinetics, several experiments were also analyzed using the general solution for the second-order reaction: $d/dt\,[\ln([R_o] - [RF])/([F_o] - [RF])] = k_f([R_o] - [RF])$, where $[F_o]$ = the initial concentration of ^{3}H-FMLP and $[R_o]$ = the initial receptor concentration.[27,38] When $[F_o] \gg [R_o]$, we may analyze this reaction as one of pseudo–first-order kinetics, that is: $d/dt\,[RF] = k_f\,[F_o][R]$. Integrating, this becomes: $\ln([R_o] - [RF]) = \ln([R_o]/[F_o]) - k_f[F_o]t$ or $d/dt\,[\ln([R_o] - [RF])] = -k_f[F_o]$.[38]

Granulocyte aggregation Granulocyte aggregation was performed as previously described.[6] Fifty microliters of ZAP or FMLP, in PBS, was added to 450 μL of a granulocyte suspension (1.1 × 10^7 cells/mL in HBSS–HSA) containing the indicated concentration of MP, which was being stirred at 37 °C at 900 rpm in a Payton 300B aggregometer/recorder system (Payton Associates, Buffalo). The resulting light transmittance was recorded on a stripchart recorder. The state of granulocyte aggregation was also confirmed by light microscopy of aliquots taken from the aggregometer

Chemotaxis. Cell migration was assayed under agarose using the technique of Nelson and co-workers.[39] Cells were allowed to migrate toward the stimulus for 2 hours in a CO_2 incubator at 37 °C to various concentrations of FMLP as indicated.

Reversal of inhibition Granulocytes (10^7 per milliliter in HBSS–HSA) were exposed to the indicated concentration of MP or PBS for 2 or 10 minutes as indicated at 22 °C and then washed by addition of 10 mL of ice-cold HBSS–BSA and centrifugation at 400 g for 5 minutes. This wash procedure was repeated as indicated, and the cells were then resuspended to an appropriate volume in HBSS or HBSS–BSA.

Receptor down-regulation and granulocyte desensitization. Granulocytes (1.25 × 10^7) were suspended in 5 mL of HBSS-HSA containing MP at the concentrations indicated The mixtures were incubated at 22 °C for 2 minutes, and FMLP (10^{-5} mol/L in PBS) was then added to a final concentration of 10^{-6} mol/L The cells were incubated at 22 °C for 20 minutes and then washed by the addition of 45 mL of 0 °C PBS–HSA containing the same concentration of MP as the incubation mixture and centrifugation at 400 g for 6 minutes They were then washed three more times as described above· twice the PBS–HSA, then once with HBSS–HSA. The cells were then suspended in HBSS–HSA at the desired concentration prior to assay. All wash procedures were performed at 0 °C.

RESULTS

Equilibrium FMLP binding. As previously described, MP inhibited, in a dose-dependent manner, FMLP binding to the granulocyte surface, whereas sodium succinate alone had no effect.[27] Examination of ^{3}H-FMLP binding at varying ^{3}H-FMLP concentrations, in the presence and absence of 0.125 mg/mL of MP, revealed no decrease in the number of surface FMLP receptors in the presence of drug (Fig 1).[27] MP reduced the apparent equilibrium binding constant by ~70% (Fig 1).[27] The receptor affinity and receptor number observed here are similar to those previously reported.[27,40] MP reduced binding with a similar dose response at 22 °C as at 4 °C (data not shown).[27,41]

Kinetics of FMLP binding. The dissociation rate constant for the peptide–receptor interaction, k_r, was determined in the presence of excess unlabeled FMLP and in the presence of 5 mg/mL of MP (data not shown). As previously

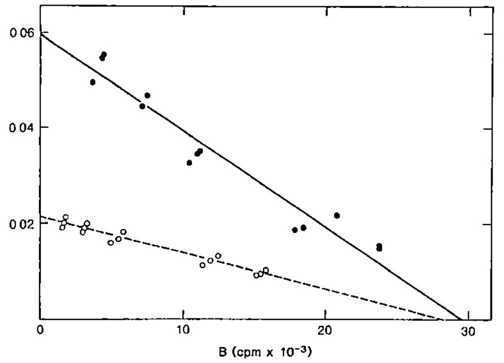

Fig 1. Methylprednisolone decreases the affinity of the f-methionine-leucine-phenylalanine (FMLP) receptor interaction without altering the number of receptors expressed on the polymorphonuclear cell (PMN) surface. Specific ^{3}H-FMLP binding to human neutrophils at varying ^{3}H-FMLP concentrations at 0 °C in the absence (—●—) and presence of 0.125 mg/mL of methylprednisolone sodium succinate (MP) (—○—). Data are plotted according to Scatchard as (B/F) *v* (B) in dpm per 5 × 10^6 cells, where B = concentration of receptor bound ^{3}H-FMLP and F = concentration of free ^{3}H-FMLP. Correlation coefficients were 0.967 (control) and 0.964 (MP). The derived K_{eq}s were 2.5 × 10^7 mol/L^{-1} (control) and 9.2 × 10^6 mol/L^{-1} (MP). The derived numbers of receptors per cell were 29,400 (control) and 28,000 (MP). Each point represents the mean of triplicate determinations. A duplicate experiment gave similar results.

reported,[27,42] not all bound ^{3}H-FMLP dissociated rapidly from the cell, possibly representing internalization of peptide, which is known to occur at 22 °C.[40,42–44] Much larger fractions of radiolabel remained cell-associated when such experiments were performed at 37 °C (data not shown), as has also been reported.[27,35,42,43,45,46] When the initial portion of the dissociation curve was corrected by subtraction of the amount of nondissociating ^{3}H-FMLP, ~85% of the total ^{3}H-FMLP bound prior to the addition of unlabeled FMLP dissociated with an apparent k_r of 1×10^{-2} s^{-1}. The time course of receptor-ligand dissociation following the addition of excess unlabeled FMLP was approximately the same as that observed following addition of MP to a final concentration of 5 mg/mL. The observed k_r (means of two separate experiments) was 1.2×10^{-2} s^{-1} in the presence of excess unlabeled FMLP. These values of k_r are comparable with those observed for the structurally similar chemotactic peptide *N*-formyl-norleucyl-leucyl-phenylalanine, to its receptor on rabbit neutrophils.[42] Analysis of association data was performed under conditions yielding pseudo-first-order kinetics in the presence of a tenfold excess concentration of peptide over receptor.[27] As previously reported, MP decreased k_f from ~2.3×10^5 (± 0.4) (SD) L/mol-s to ~0.8×10^5 L/mol-s.

Reversibility of inhibitor effect. When granulocytes were incubated with 2 mg/mL of MP and then washed three times with HBSS–BSA, the inhibition of aggregation as well as the blockade of FMLP binding were both reversed (Figs 2 and 3). Granulocyte aggregation induced by FMLP (Fig 2) or by activated complement (not shown) was inhibited by 2 mg/mL of MP, as previously described.[1] When these cells were washed three times in HBSS–BSA to remove the drug, the addition of FMLP to a stirred suspension of granulocytes in an aggregometer produced a wave of aggregation like that of control granulocytes not exposed to drug (Fig 2).

FMLP binding to granulocytes was markedly inhibited by 1.5 mg/mL of MP. When these granulocytes were incubated with drug for 2 minutes at 22 °C and then washed three times with PBS–BSA at 0 °C, FMLP binding was similar to that of control cells incubated with PBS (Fig 3). Similar reversibility of drug effect occurred when cells were preincubated with drug for 30 minutes at 22 °C (data not shown).

Down-regulation and desensitization. We next tested the effects of MP on FMLP receptor down-regulation and granulocyte desensitization. Neutrophils exposed to FMLP become desensitized to further stimulation with FMLP.[9,42,44,47–51] This desensitization is accompanied by a down-regulation of the number of receptors for FMLP present on the surface of the cells. We confirmed these observations by treating neutrophils with either PBS or MP for 2 minutes at 22 °C and then incubating the cells with 10^{-6} mol/L of FMLP in the presence of drug or buffer. The cells were then washed as described in the Materials and Methods section, resuspended in HBSS–HSA, and tested for surface

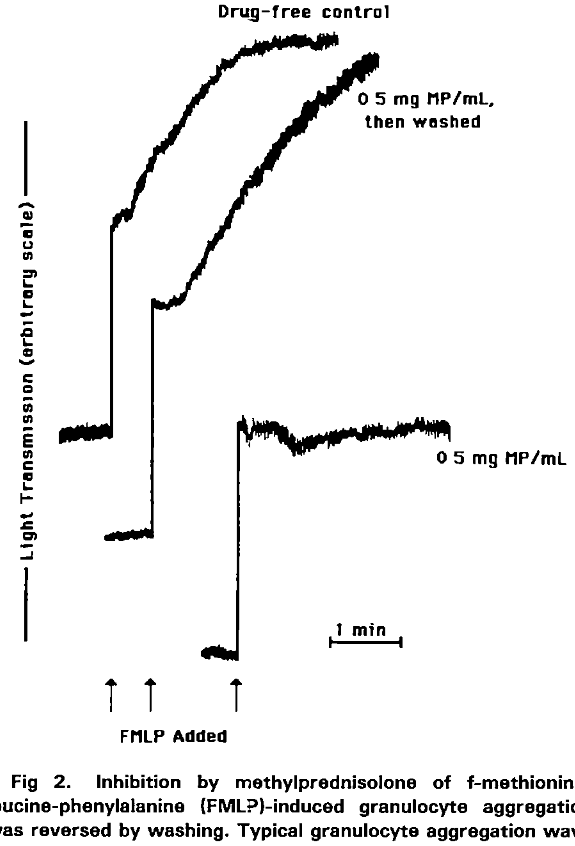

Fig 2. Inhibition by methylprednisolone of f-methionine-leucine-phenylalanine (FMLP)-induced granulocyte aggregation was reversed by washing. Typical granulocyte aggregation wave showing inhibition of aggregation in the presence of 2 mg/mL of MP; when cells previously exposed to 2 mg/mL of MP for 10 minutes at 22 °C and then washed to remove MP were stimulated with 10^{-7} mol/L of FMLP, the resulting aggregation wave resembled that of untreated control cells. Change in light transmittance (ΔT) is plotted *v* time. As in previous studies, changes in light transmittance have been documented to accompany changes in aggregation state.

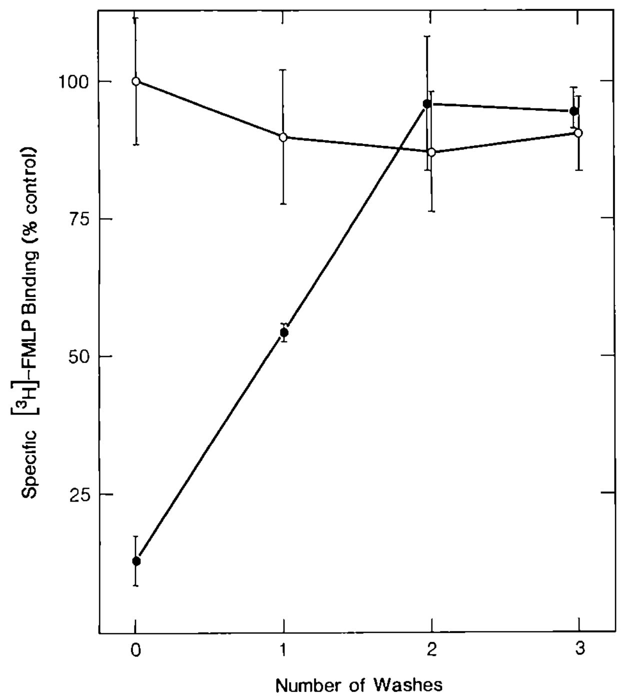

Fig 3. Inhibition by methylprednisolone of ^{3}H-f-methionine-leucine-phenylalanine (FMLP) binding to granulocytes is reversed by washing. Granulocytes were incubated with phosphate-buffered saline (PBS) (○) or 1.5 mg/mL of MP (●) for 2 minutes at 22 °C. Initially, and after washing the cells 1, 2, or 3 times with ice-cold Hanks' buffered salt solution–bovine serum albumin (HBSS–BSA), the cells were again assayed for ^{3}H-FMLP binding at 0 °C as described in the text. Specific ^{3}H-FMLP binding per cell is shown as percentage of binding observed initially in the absence of drug. ^{3}H-FMLP was 18 nmol, and there were 3 × 10^6 granulocytes per tube. Each point represents the mean ± SD of triplicate samples. A duplicate experiment gave similar results.

FMLP receptors and for aggregation in response to FMLP. As expected, neutrophils exposed to FMLP in the absence of drug demonstrated both desensitization and FMLP receptor down-regulation (Fig 4). Neutrophils exposed to FMLP in the presence of MP (Fig 4) also demonstrated receptor down-regulation and desensitization. These same concentrations of MP were shown in separate experiments to inhibit granulocyte aggregation markedly in response to 10^{-7} mol/L of FMLP, however (data not shown).[1,52,53] This observation was not limited to aggregation as shown in Table 1. Chemotactic desensitization also occurred under these same conditions. Methylprednisolone reversibly inhibited granulocyte chemotaxis in a dose-dependent manner. Cells preincubated with FMLP in the presence of MP and then washed were desensitized to further FMLP stimulation when response was measured by chemotaxis as well as by aggregation.

DISCUSSION

Corticosteroids are widely used for their antiinflammatory effects and have been suggested to be of benefit in various disorders, including endotoxic shock and the adult respiratory distress syndrome.[1,11,12,18,20–22,54,55] These benefits, however, have been reported to occur only when the drugs are administered in doses much larger [eg, 30 mg/kg intravenously (IV) for MP] than those conventionally used to achieve an antiinflammatory effect.[17,18,54]

It would seem plausible that the effects of these drugs at such high doses might well be different from those observed with conventional low doses. We previously reported that such high concentrations of corticosteroids (0.1 to 1 mg/mL) inhibit binding of the chemotactic peptide FMLP to its receptor on the granulocyte surface and that the dose–response relationship of the inhibition of FMLP binding

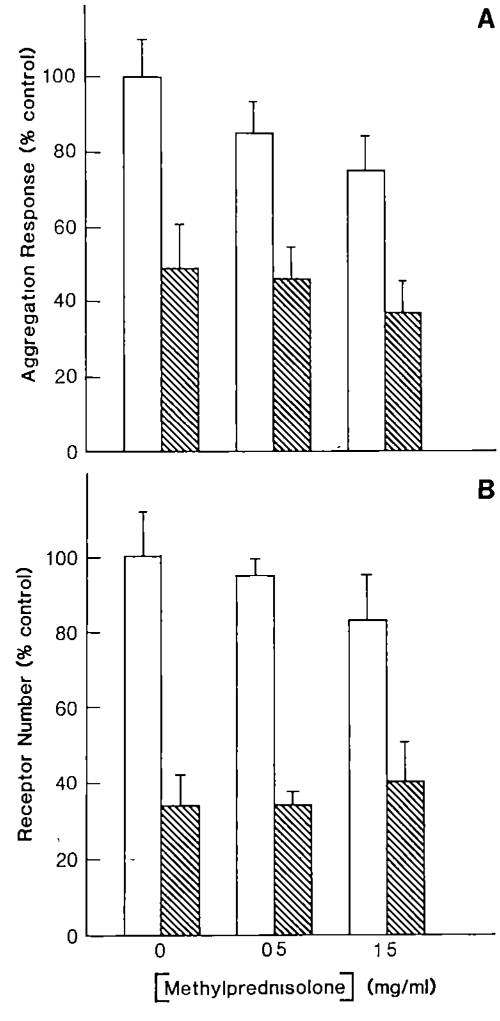

Fig 4. Neutrophils exposed to f-methionine-leucine-phenylalanine (FMLP) in the presence of methylprednisolone sodium succinate (MP) became desensitized to further FMLP stimulation and down-regulated their surface FMLP receptors. Neutrophils were incubated in the presence of MP at the concentration indicated for 2 minutes at 22 °C and subsequently for 20 minutes at 22 °C in the presence of the same concentration of MP in addition to 10^{-8} mol/L of FMLP (hatched bars) or buffer (open bars). The cells were washed and assayed for surface FMLP receptors and aggregation response to 10^{-7} mol/L of FMLP as described in the text. (A) Aggregation response is reported as the height of the aggregation curve at 3 minutes expressed as percentage of control. (B) FMLP receptor number is reported as specific FMLP binding per cell in the presence of 120 nmol/L of ^{3}H-FMLP and is expressed as percentage of control. Each point is the mean ± SD of four separate experiments, each of which was performed in triplicate.

Table 1. Chemotactic Deactivation of Neutrophils to FMLP

Pretreatment	Chemotactic Index (Percentage of Random Migration)
Buffer	372 ± 22
Buffer + 0.5 mg/mL of MP	346 ± 26
Buffer + 1 5 mg/mL of MP	375 ± 48
Buffer + FMLP	179 ± 21
Buffer + 0 5 mg/mL of MP + FMLP	192 ± 5
Buffer + 1 5 mg/mL of MP + FMLP	220 ± 45

Neutrophils exposed to f-methionine-leucyl-phenylalanine (FMLP) in the presence of methylprednisolone sodium succinate (MP) became desensitized to further FMLP stimulation Neutrophils were incubated in the presence or absence of MP at the concentration indicated for 2 minutes at 22 °C and subsequently for 20 minutes at 22 °C in the presence of the same concentration of MP in addition to 10^{-6} mol/L of FMLP or buffer. The cells were then washed, and chemotaxis was assayed under agarose as described in the text. Results are reported as chemotactic index (direct/random migration) when cells migrated toward a well containing 10^{-6} mol/L of FMLP. Each value is the mean ± SEM of four separate determinations In each case, the chemotactic index of cells preincubated with FMLP in the presence or absence of MP was significantly lower than that of cells preincubated with buffer alone [$P <$ 001 for (MP) = 0 or 0 5 mg/mL, $0.01 < P < .05$ for (MP) = 1.5 mg/mL, unpaired *t* test], whereas the chemotactic index of cells preincubated in MP alone was not statistically different from that of control cells ($P > .2$) When MP was not removed by washing, chemotaxis was inhibited in a dose-dependent manner with a potency similar to that observed with aggregation.

parallels the inhibition of granulocyte responses to FMLP.[27] We used FMLP as a probe of granulocyte receptor–ligand interaction because previous in vitro and in vivo studies of granulocyte responses to chemotactic stimuli suggested that FMLP was a reasonable model for C5a-induced granulocyte leukostasis and tissue injury and because radiolabeled FMLP is readily available.[9,27,29,42,56]

Our current finding that these effects of high-dose corticosteroids can be removed by washing may explain the observation of other researchers that short courses of high-dose corticosteroids do not result in very marked effects on neutrophil chemotaxis.[32] This observation led us to question whether these drugs might be capable of preventing the cellular response to a stimulus, while allowing a gradual increase in receptor occupancy with concomitant receptor down-regulation and desensitization. Such a phenomenon may partially explain how intermittent high doses of these drugs, which maintain high plasma concentrations for only a short time,[25,31] might exert a beneficial effect in some clinical situations. It is well known that treatment of neutrophils with chemotactic factors can result in a loss of their ability to respond to further stimulation, a phenomenon known as deactivation.[42,44,47,49–51,57–59] This deactivation may be due in part to receptor down-regulation, but other mechanisms may also be involved.[44] Neutrophils exposed to FMLP in the absence of divalent cations do not aggregate.[50] Cells preincubated with FMLP in the absence of divalent cations for >4 minutes aggregated minimally after addition to divalent cations,[50] suggesting that binding of FMLP to its receptor results in a transient ability of the cell to respond. In the absence of divalent cations, the cells do not aggregate in response to FMLP, but do become "desensitized" to further stimulation.[50] Indeed, we found concentrations of MP capable of inhibiting granulocyte response to FMLP that did allow a slowed increase in FMLP receptor–ligand binding with concomitant desensitization and receptor down-regulation. The mechanism by which corticosteroids exert their effect on the receptor–ligand interaction remains unknown.[27]

We conclude that high-dose corticosteroids can inhibit granulocyte responses to chemotactic stimuli. These same concentrations of corticosteroids inhibit binding of the synthetic chemotaxin ^{3}H-FMLP to its specific receptor on the granulocyte surface. This inhibition of binding is due primarily to a decrease in the association rate constant for peptide–receptor interaction without a significant change in dissociation rate or receptor number. A marked effect on the FMLP–receptor interaction is seen at drug concentrations approximating the plasma concentration immediately after a 30 mg/kg IV bolus, and a prominent effect is seen even at a tenfold-lower concentration. Alteration of cellular receptors may therefore be an important mechanism of action of corticosteroids on granulocyte aggregation. The observation that the effects of MP on granulocyte aggregation and FMLP receptor activity are reversed by washing, coupled with the short half-life of high doses of these drugs, explains the results of earlier studies on granulocyte function following pulse MP therapy in vivo.[32] Furthermore, the data suggest a novel mechanism of drug action; inhibiting the association kinetics of receptor–ligand interaction resulting in inhibition of cellular response, yet allowing receptor down-regulation and desensitization to further simulation. Although the clinical importance of this phenomenon remains to be shown, alteration of receptor–ligand interactions may contribute to the reported efficacy of high doses of these drugs in leukostatic and other syndromes.

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REFERENCES

1. Hammerschmidt DE, White JG, Craddock PR, Jacob HS Corticosteroids inhibit complement-induced granulocyte aggregation: A possible mechanism for their efficacy in shock states. J Clin Invest 63:798, 1979

2. Hammerschmidt DE, Weaver LJ, Hudson LD, Craddock PR, Jacob HS: Association of complement activation and elevated plasma-C5a with adult respiratory distress syndrome Lancet 1:947, 1980

3. Hohn DC, Meyers AJ, Gherini ST, Beckmann A, Markison RE, Chung AM. Production of acute pulmonary injury by leukocytes and activated complement Surgery 88:48, 1980

4 Hosea S, Brown E, Hammer C, Frank M. Role of complement activation in a model of adult respiratory distress syndrome. J Clin Invest 66:375, 1980

5. Greenberg CS, Hammerschmidt DE, Craddock PR, Jacob HS: Atheroma cholesterol activates complement and aggregates

granulocytes: Possible role in ischemic manifestations of atherosclerosis. Trans Assoc Am Physicians 92:130, 1979

6. Craddock PR, Hammerschmidt DE, White JG, Dalmasso AP, Jacob HS: Complement (C5a)-induced granulocyte aggregation *in vitro:* A possible mechanism of complement-mediated leukostasis and leukopenia. J Clin Invest 60.260, 1977

7. Craddock PR, Fehr J, Brigham KL, Kronenberg RS, Jacob HS: Complement and leukocyte-mediated pulmonary dysfunction in hemodialysis. N Engl J Med 296 769, 1977

8. Craddock PR, Fehr J, Dalmasso AP, Brigham KL, Jacob HS. Hemodialysis leukopenia Pulmonary vascular leukostasis resulting from complement activation by dialyzer cellophane membranes J Clin Invest 59 879, 1977

9. Skubitz KM, Craddock PR: The reversal of hemodialysis granulocytopenia and pulmonary leukostasis: A clinical example of selective down-regulation of granulocyte responses to $C5a_{desarg}$. J Clin Invest 67:1383, 1981

10. Pickard RN, O'Rourke RA, Crawford MH, Grover FS, McManus LM, Ghidoni JJ, Storrs SB, Olson MS: Complement localization and mediation of ischemic injury in baboon myocardium. J Clin Invest 66:1050, 1980

11 Sneiderman CA, Wilson JW: Effects of corticosteroids on complement and the neutrophilic polymorphonuclear leukocyte. Transplant Proc 7:41, 1975

12. O'Flaherty JT, Craddock PR, Jacob, HS: Mechanism of anti-complementary activity of corticosteroids *in vivo:* Possible revelance in endotoxin shock. Proc Soc Exp Biol Med 154.206, 1977

13. White GL, Archer LT, Beller BK, Hinshaw LB: Increased survival with methylprednisolone treatment in canine endotoxin shock. J Surg Res 25:357, 1978

14. Schumer W: Steroids in the treatment of clinical septic shock. Ann Surg 184·333, 1978

15. Fine J, Palmerio C, Rutenberg S: New developments in therapy of refractory traumatic shock. Arch Surg 96 163, 1968

16 Kusajima K, Wax SD, Webb WR Effects of methylprednisolone on pulmonary microcirculation. Surg Gynecol Obstet 139.1, 1974

17. Sladen A: Methylprednisolone. Pharmacologic doses in shock lung syndrome. J Thorac Cardiovasc Surg 71:800, 1976

18. Lillehei RC, Dietzman RH, Motsay GJ, Beckman CB, Romero LH, Shatney CH: Growth of the concept of shock and review of present knowledge, in Glenn TM (ed): Steroids and Shock. Baltimore, University Park Press, 1974, p 377

19. Wilson JW: Treatment or prevention of pulmonary cellular damage with pharmacologic doses of corticosteroids. Surg Gynecol Obstet 134.675, 1971

20 Greisman SE: Experimental gram-negative bacterial sepsis: Optimal methylprednisolone requirements for prevention of mortality not preventable by antibiotics alone. Proc Soc Exp Biol Med 170:436, 1982

21. Hinshaw LB, Archer LT, Beller-Todd BK, Coalson JJ, Flournoy DJ, Passey R, Benjamin B, White GL: Survival of primates in LD_{100} septic shock following steroid/antibiotic therapy. J Surg Res 28:151, 1980

22. Hinshaw LB, Beller-Todd BK, Archer LT. Current management of the septic shock patient: Experimental basis for treatment. Circ Shock 9.543, 1982

23. Oredugba O, Mazumdar DC, Meyer JS, Lubowitz H· Pulse methylprednisolone therapy in idiopathic, rapidly progressive glomerulonephritis Ann Intern Med 92.504, 1980

24 Liebling MR, Leib E, McLaughlin K, Blocka K, Furst DE, Nyman K, Paulus HE· Pulse methylprednisolone in rheumatoid arthritis. Ann Intern Med 94·21, 1981

25. Proceedings of a symposium: Motrin (ibuprofen) past, present, and future. Am J Med 77:1, 1984

26. Kimberly RP, Lockshin MD, Sherman RL, McDougal JS, Inman RD, Christian CL. High-dose intravenous methylprednisolone pulse therapy in systemic lupus erythematosus. Am J Med 70·817, 1981

27. Skubitz KM, Craddock PR, Hammerschmidt DE, August JT: Corticosteroids block binding of chemotactic peptide to its receptor on granulocytes and cause disaggregation of granulocyte aggregates in vitro. J Clin Invest 68:13, 1981

28. Williams LT, Snyderman R, Pike MC, Lefkowitz RJ Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes Proc Natl Acad Sci USA 74·1204, 1977

29 O'Flaherty JT, Showell HJ, Ward PA· Neutropenia induced by the systemic infusion of chemotactic factors. J Immunol 118:1586, 1977

30 Garg DC, Ng P, Weidler DJ, Sakmar E, Wagner JG. Preliminary *in vitro* and *in vivo* investigations on methylprednisolone and its acetate. Res Commun Chem Pathol Pharmacol 22:37, 1978

31. Axelrod L Glucocorticoid therapy Medicine 55:39, 1976

32 Perez HD, Kimberley RP, Kaplan HB, Edelson H, Inman RD, Goldstein IM· Effect of high-dose methylprednisolone infusion on polymorphonuclear leukocyte function in patients with systemic lupus erythematosus. Arthritis Rheum 24:641, 1981

33 Boyum A: Isolation of mononuclear cells and granulocytes from blood Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation of 1 × g. Scand J Clin Lab Invest 21:77, 1968 (suppl 97)

34. Delisi C· The effect of cell size and receptor density on ligand-receptor reaction rate constants. Mol Immunol 18:507, 1981

35. Zigmond SH, Sullivan SJ, Lauffenburger DA: Kinetic analysis of chemotactic peptide receptor modulation. J Cell Biol 92:34, 1982

36 DeLisi C, Wiegel FW· Effect of nonspecific forces and finite receptor number on rate constants of ligand-cell bound-receptor interactions. Proc Natl Acad Sci USA 78:5569, 1981

37 Scatchard G: The attraction of proteins for small molecules. Ann NY Acad Sci 51·660, 1949

38 Moore WJ. Physical Chemistry. Englewood Cliffs, NJ, Prentice-Hall. 1972, pp 329–344

39 Nelson RD, Quie PG, Simmons RL: Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J Immunol 115 1650, 1975

40 Niedel J, Wilkinson S, Cuatrecasas P. Receptor-mediated uptake and degradation of ^{125}I-chemotactic peptide by human neutrophils. J Biol Chem 254:10700, 1979

41. Skubitz KM, Hammerschmidt DE· Effects of ibuprofen on chemotactic peptide-receptor binding and granulocyte response. Biochem Pharmacol (in press)

42. Sullivan SJ, Zigmond SH· Chemotactic peptide receptor modulation in polymorphonuclear leukocytes J Cell Biol 85:703, 1980

43. Niedel JE, Kahane I, Cuatrecasas P: Receptor-mediated internalization of fluorescent chemotactic peptide by human neutrophils. Science 205 1412, 1979

44. Vitkauskas G, Showell HJ, Becker EL Specific binding of synthetic chemotactic peptides to rabbit peritoneal neutrophils· Effect on dissociability of bound peptide, receptor activity and subsequent biologic responsiveness (deactivation) Mol Immunol 17:171, 1980

45. Jesaitis AJ, Naemura JR, Painter RG, Sklar LA, Cochrane CG The fate of an *N*-formylated chemotactic peptide in stimulated human granulocytes: subcellular fractionation studies J Biol Chem 258:1968, 1983

46. Jesaitis AJ, Naemura JR, Sklar LA, Cochrane CG, Painter RG: Rapid modulation of *N*-formyl chemotactic peptide receptors on the surface of human granulocytes Formation of high-affinity

ligand-receptor complexes in transient association with cytoskeleton. J Cell Biol 98.1378, 1984

47. Simchowitz L, Atkinson JP, Spilberg I: Stimulus-specific deactivation of chemotactic factor-induced cyclic AMP response and superoxide generation by human neutrophils. J Clin Invest 66:736, 1980

48 Nelson RD, McCormack RT, Fiegel VD, Herron M, Simmons RL, Quie PG: Chemotactic deactivation of human neutrophils: Possible relationship to stimulation of oxidative metabolism. Infect Immun 23:282, 1979

49. Seligmann BE, Fletcher MP, Gallin JI: Adaptation of human neutrophil responsiveness to the chemoattractant *N*-formylmethionylleucylphenylalanine. J Biol Chem 257.6280, 1982

50. O'Flaherty JT, Kreutzer DL, Showell HS, Becker EL, Ward PA: Densensitization of the neutrophil aggregation response to chemotactic factors. Am J Pathol 93:693, 1978

51. Donabedian H, Gallin JI: Deactivation of human neutrophil chemotaxis by chemoattractants: Effect on receptors for the chemotactic factor f-met-leu-phe. J Immunol 127:839, 1981

52. Hammerschmidt DE, Flynn PJ, Coppo PA, Skubitz KM, Jacob HS: Synergy among agents inhibiting granulocyte aggregation. Inflammation 6:169, 1982

53. Flynn PJ, Becker WK, Vercellotti GM, Weisdorf DJ, Craddock PR, Hammerschmidt DE, Lillehei RC, Jacob HS· Ibuprofen inhibits granulocyte responses to inflammatory mediators. Inflammation 8.33, 1984

54. Wise WC, Cook JA, Eller T, Halushka PV: Ibuprofen improves survival from endotoxic shock in the rat. J Pharmacol Exp Ther 215:160, 1980

55. Jacobs ER, Soulsby ME, Bone RC, Wilson FJ Jr, Hiller FC: Ibuprofen in canine endotoxic shock. J Clin Invest 70:536, 1982

56. O'Flaherty JD, Kreutzer DL, Showell HJ, Vitkauskas G, Becker EL, Ward PA: Selective neutrophil desensitization to chemotactic factors. J Cell Biol 80:564, 1979

57. Ward PA, Becker EL: The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. J Exp Med 127:693, 1968

58. Sklar LA, Jesaitis AJ, Painter RG, Cochrane CG· The kinetics of neutrophil activation. The response to chemotactic peptides depends upon whether ligand-receptor interaction is rate-limiting. J Biol Chem 256:9909, 1981

59. Lefkowitz RJ, Sibley DR: Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. Nature 317:124, 1985

What Should Be the Morphologic Criteria for the Subdivision of Follicular Lymphomas?

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The members of the Pathology Panel for Lymphoma Clinical Studies undertook a collaborative study with the hope of resolving some of the controversies regarding the criteria and methods for the subclassification of follicular lymphomas (FLs). A group of 105 patients with FL were subclassified by seven hematopathologists according to two methods. In the first method, cases were subclassified according to the Rappaport, Lukes and Collins, and Working Formulation systems. In each of these systems, FLs are subclassified by estimation of the different cell populations, without actual counting of cells. In the second method, precise counts of different cells were made according to the standard and modified Berard methods. With this counting method, diagnoses were independently derived, based on counts provided by the seven pathologists, for large cleaved (LC), small noncleaved (SNC), and large noncleaved (LNC) cells. To ascertain what method and which criteria are most useful in predicting survival, we made clinicopathologic correlations. When the subjective (first method) diagnoses were rendered, and when the consensus diagnoses of the seven pathologists were used, there were no significant differences in survival among patients with the different subtypes. On the other hand, when we used the counting method of Berard (second method) and the cut-off points for the cell counts suggested by him for the subclassification, we were able to divide the patient population into prognostic subgroups. Because the cut-off points proposed by Berard are not derived objectively, we made statistical comparisons of survival curves to determine cut-off points (and thus to establish objective criteria). We found that the patient population could be separated into at least two prognostic groups, for SNC and/or LNC and for SNC + LNC + LC cells. The cut-off points which we derived differed with cell type, however. Until the usefulness of these new cut-off points is established, we recommend that the cut-off points and the counting method of Berard be used for the subclassification of FL. Because the choice of treatment for the different subtypes of FL is totally dependent on the histologic diagnosis, and because of the variability among the diagnoses of pathologists, treatment planning is difficult.

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FOLLICULAR (nodular) lymphomas (FLs) are subdivided morphologically into three cell types, small cleaved, large cell, and mixed (small cleaved and large cell).[1] This subclassification, which usually is based on the subjective assessment of the percentages of small and large lymphoid cells ("histiocytes"), is important clinically, because major therapeutic decisions are based on it.[2-7] There is general agreement that nodular, poorly differentiated lymphocytic (NPDL) lymphoma is a low-grade malignant lymphoma,[2-8] and that nodular "histiocytic" (NH) lymphoma is of an intermediate grade[9-10]; however, there is disagreement regarding the clinical behavior of nodular mixed (NM) lymphoma.[2,3,11-13] In a study by the National Cancer Institute, patients with NM had longer survival times than did patients with NPDL lymphoma.[12] Results obtained at Stanford University[3] suggest that the NM type has a prognosis similar to that of NPDL, whereas the results of the Eastern Cooperative Oncology Group[13] indicate that the survival of patients with NM is significantly shorter than that observed in NPDL lymphoma. These conflicting data may be a result of disagreement among pathologists as to the number of large cells that are necessary for classification of a lymphoma as either the mixed cell type or the large lymphoid ("histiocytic") type.

There are not only differences in the morphologic criteria for the subclassification of follicular lymphomas, but also in the methods used for determining the number of large cells In the Rappaport,[2] Lukes and Collins,[14] and Working Formulation[1] systems, the subclassification of FLs is subjective, based on estimates of the percentages of large cells within follicles and not on actual counting of cells. In the Berard method,[13,15] on the other hand, actual counts of large noncleaved cells (LNC) are made and their number determine a subclassification. In the Working Formulation study, in which the Lukes and Collins classification was used, it was found that FLs of the small noncleaved (SNC) cell type had the most aggressive natural history among the FLs; they are placed in the high-grade category.[16] These data suggest that increased numbers of SNC cells adversely affect survival.

In view of these considerations, we concluded that actual counting of large cleaved (LC), LNC, and SNC cells would be the optimal method of determining the precise criteria for the subclassification of FLs. Moreover, we believed that these criteria should not be based on preconceived ideas

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regarding the cut-off points for the number of large cells, but that the cut-off points should be derived objectively on the basis of statistical comparisons of survival curves.

The current study, in which we set out to ascertain the precise criteria for subdivision of FLs, is based on a patient population taken from the Southwest Oncology Group (SWOG). All patients were treated uniformly and intensively with curative intent, and all were followed up for long periods (SWOG protocol numbers 7426 and 7713).[17,18] The specific objectives of our study were: (a) to determine which parameters independently influenced survival, (b) to ascertain survival times based on subjective diagnosis (based on estimated counts), (c) to ascertain survival times based on actual counts by using two methods, and (d) to assess the relative value of the subjective and objective (counting) methods in predicting survival times.

MATERIALS AND METHODS

Histologic material from 105 patients with FL who had been treated previously in SWOG studies (nos 7426 and 7713) was reviewed by seven hematopathologists The details of patient selection have been described previously.[19] In general, these patients were chosen for review because adequate histologic material was available, patient follow-up was >5 years, all had advanced disease (stage III or IV), and all had been treated uniformly with combination chemotherapy that included cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) [17,18]

The SWOG diagnosis of record is a refined diagnosis. The initial diagnosis is made by the local institution's pathologist. A second review is made by the pathologists of the SWOG regional center, and a third review is made by the Repository Center for Lymphoma Clinical Studies.[20,21] If the diagnoses by the regional center and by the Repository Center agree, they constitute the SWOG diagnosis of record. If the diagnoses disagree, the case is reviewed by the Pathology Panel, and its diagnosis is the SWOG diagnosis of record.[21]

A trial review session was held at the Repository Center at City of Hope National Medical Center on an 11-headed microscope At this session, the design of the study and the morphologic criteria to be used were discussed A series of cases were reviewed, and a consensus was obtained on the criteria for identification of small cleaved, LC, SNC, and LNC cells. The statistician for the study (G.E.M.), participated in this review to ensure that the research design was statistically adequate and that uniform standards were developed for the study. The published criteria of Lukes and Collins were circulated among the participants and, after extensive discussion, each pathologist was at liberty to use whatever criteria he or she felt were most useful for distinguishing the different cell types

All participants had an American Optics microscope with lenses having identical magnifications. Everyone had a WF10X eyepiece, and a 40x high-magnification lens. The field of view index was 18, and the diameter was 4.58 mm. The high-power field (HPF) for every pathologist was the same

Rules and Criteria for Rendering of Diagnoses According to the Estimation Method

In the estimation method, FLs were subclassified according to cell type by estimation of different cell populations, without any attempt to count cells. Cases were diagnosed according to the Rappaport,[2] Lukes and Collins,[14] and Working Formulation[1] systems. To ensure consistency among the pathologists, we agreed on the following criteria for the rendering of diagnoses based on the three systems

1 For the Rappaport classification,[2] a case was categorized as NPDL when the estimated percentage of large cells was <25%. If the estimate for large cells was between 25% and 50%, the case was classified as NM; if the percentage of large cells was considered >50%, the case was classified as NH lymphoma. Rare cases were classified as undifferentiated when the majority of cells were interpreted as undifferentiated

2 For the Lukes and Collins classification,[14] a case was considered to be of the SC cell type when the predominant population was of the SC cell type. A case was classified as LC cell type when SC cells were mixed with LC and LNC cells; however, in this category, the estimate for LNC cells was always <25%. When the estimate for LNC cells was >25%, the case was classified as LNC cell type. A case was termed SNC cell type when SNC cells were the predominant cell population.

3 For the Working Formulation,[1] a case was classified as small cleaved cell type when the estimated percentage of large cells was <25%. A case was classified as mixed-cell type when there was a mixture of small cleaved, LC, and LNC cells. If it was estimated that ≥50% of the cells were large cells, a case was classified as large-cell type A case was considered to be of the SNC cell type when SNC cells predominated.

Rules and Criteria for Counting and Derivation of Diagnosis

Standard Berard counting method. The standard Berard[13,15] method of deriving diagnoses is based on counts of LNC cells, with counts of 0 to 5 LNC cells per HPF giving a diagnosis of PDL; >5 to 15 LNC cells per HPF, NM; and >15 LNC cells per HPF, NH lymphoma.

Modified Berard method In this study, we modified the Berard method[19] in two ways: (a) Rather than counting LNC cells only, we applied the cut-off points of 5 and 15 cells to SNC cells only, to SNC + LNC cells combined, and to SNC + LNC + LC cells; and (b) we explored whether other numbers of cell counts provided better cut-off points with application to counts of LNC, SNC, SNC + LNC, and SNC + LNC + LC cells.

In addition, counts of the three cell types (SNC, LNC, LC) were made up to 50 cells. The pathologists did the counting only and did not translate the counts into diagnoses The statistician (a) objectively derived diagnoses by using the 5-cell and 15-cell cut-off points, and (b) computed survival curves to obtain natural cut-off points.

Reasons for modifying the standard Berard method 1 In the Rappaport classification, the nodular lymphomas are subdivided according to the subjectively determined relative number of large cells (histiocytes) Since the development of the Rappaport system, Berard,[13,15] in his practice, has made precise counts of only LNC cells to subdivide follicular lymphomas, because he has found that counting of these cells is a more objective and reliable method than subjective estimation.

2 In the Lukes and Collins classification,[14] on the other hand, FLs are subdivided based on the relative numbers of LC, SNC, and LNC cells; theirs is also a subjective method of estimation

3 In the Working Formulation study,[1] it was found that follicular lymphomas of the SNC cell type had the most aggressive natural history, and these lymphomas were placed in the high-grade category.[1,16] The data from NCI studies suggest that increased numbers of SNC cells adversely affect survival. Therefore, we considered it important to include counts of these cells.

4 Rather than retaining current values of the cut-off point for the number of large cells required for the subclassification of follicular lymphomas, we decided that the criteria for cut-off points should be derived based on correlations of survival.

For each of the 105 cases, therefore, each of the seven pathologists was required to count and record the number of LC, LNC, and SNC cells in a total of 10 HPF. The entire slide was screened, and only those follicles identified by the pathologist as showing a maximum of LC, SNC, and LNC cells were used for counting. The counts that are used in this study are the initial counts provided by the pathologists, and the results have been published.[19] In 97 cases, at least four pathologists provided counts; therefore, in some cases, the average of 40 per HPF was used, whereas in others, the average of 70 per HPF was used.

To reach a diagnosis, we applied the standard and modified Berard criteria to the sum of the averages across 10 HPF of the three cell groups (LC, LNC, and SNC) for each case for each pathologist. Small noncleaved cells were included among the large cells because, with respect to nuclear size and the presence of distinct nucleoli, the SNC cells bore a greater resemblance to LNC cells than to small cleaved cells. In addition, the same determinations were made according to the standard Berard method, based only on counts of LNC cells.

Statistical Methods

Kaplan-Meier[22] curves were computed and Mantel-Cox[23] tests done for comparison of survival curves. Some of the comparisons had relatively low power for detecting a significant difference because of the small size of some subgroups and the small numbers of deaths in some of these subgroups. We used Cox regression[24] models to examine the effect of potential prognostic variables on survival. Survival was calculated from the date when treatment was started until death or to the date of the last follow-up

RESULTS

Parameters Influencing Survival

We derived Cox regression[24] models for the diagnosis by each pathologist, for the consensus (the diagnosis agreed upon by four or more pathologists on initial evaluation or review), and for the SWOG diagnosis to determine which variables predicted survival times (Table 1). Variables considered in the step-up procedures were age, pathologic stage, sex, cell counts (LNC, SNC, and LC), and subjective diagnosis; those variables with a significance level <0.10 were included in the regression equation. When the Cox regression analysis was carried out for each pathologist, for the consensus, and for the SWOG diagnosis, patient age and pathologic stage were always important predictors of survival, with increased age and higher stage being associated with a poorer prognosis. As determined by univariate statistical analysis, the median survival of the 46 patients with stage III disease was significantly longer than that noted for the 59 patients with stage IV disease ($P = .049$). In stage III group, 38 patients (83%) obtained a complete remission (CR), whereas only 36 patients (61%) in stage IV group obtained a CR. The age range of our 105 patients was 20 to 85 years, with a mean of 54.1 years. Using a univariate analysis, patients ≥54 years of age had a shorter survival than those <54 years of age ($P = .03$). Patients ≥60 years of age had a shorter survival than did those <60 years of age ($P = .01$); and patients ≥70 years of age had a shorter survival than did those <70 years of age ($P = .00001$). For three pathologists (B, C, and F), the number of SNC cells counted was also predictive; a greater number was associated with a poorer prognosis. For three pathologists (A, D, and G), the diagnosis of a "histiocytic" lymphoma was predictive of shorter survival. For pathologist E, neither counts nor subjective diagnosis contributed significantly to the prediction of survival. For the consensus diagnosis, the number of LNC cells was important, higher numbers being associated with a poorer prognosis. For the SWOG diagnosis, the presence of a mixed cell lymphoma was associated with shorter survival. (For the SWOG diagnosis, cell counts were not done.)

Table 1. Cox Regression Analysis of Survival (P values listed are those for variables for which $P < 0\ 10$)

				Cell Counts			Rappaport Diagnosis	
Pathologist	Age	Pathol. Stage	Sex	LNC	SNC	LC	M	H
A	006	.008	—	—	—	—	—	.024
B	028	.024	—	—	017	—	—	—
C	041	.022	—	—	085	—	—	—
D	060	.028	—	—	—	—	—	066
E	.027	.032	—	—	—	—	—	—
F	031	.013	—	—	.016	—	—	—
G	.028	.019	—	—	—	—	—	.041
Consensus	.044	008	—	.086	—	—	—	—
SWOG	.085	.008	—	NA	NA	NA	.001	—

Abbreviations. LNC, large noncleaved; SNC, small noncleaved; LC, large cleaved; M, mixed; H, "histiocytic"; NA, not applicable

Survival Based on Subjective Diagnosis

Our previous study showed that there was great variability among the seven pathologists with respect to their subjective diagnosis and the actual cell counts.[19] Therefore, it seemed appropriate to use only the consensus diagnosis and the overall averages of counts of the various cell types.

When we used the consensus Rappaport diagnosis to subclassify FLs into three groups, we obtained no significant difference in survival among these groups (Fig 1) ($P = .33$). The results were similar with the Lukes and Collins classifi-

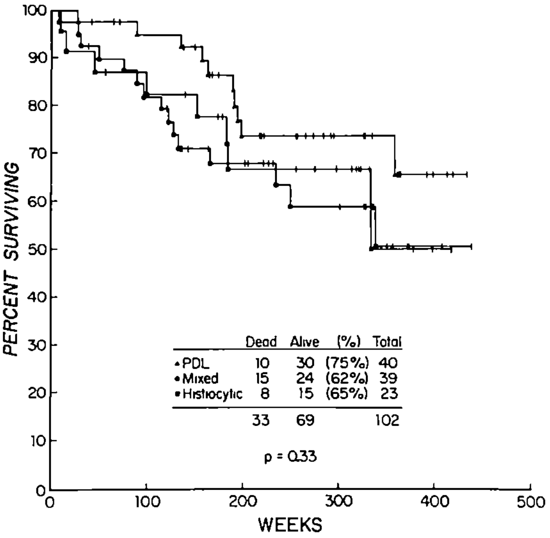

Fig 1. There were no significant differences in survival times among the three morphologic subtypes of follicular lymphomas when the consensus diagnoses (subjective) were used ($P = .33$).

cation ($P = .15$) and the Working Formulation ($P = .17$). In contrast, when we used the SWOG histologic diagnosis of record to generate survival curves, we found a highly significant difference (Fig 2) ($P = .001$).

Correlation of Survival With Derived Diagnosis

Using the standard Berard cut-off points of 0 to 5 LNC cells for PDL, >5 to 15 LNC cells for mixed, and >15 LNC cells for histiocytic lymphoma, we generated survival curves based on LNC cells (Fig 3). The cell count used was the average of the counts across 70 HPF (ie, 10 HPF for each pathologist for each case), which showed marginally significant differences among the cell types ($P = .07$). We applied these same cut-off points to SNC (Fig 4), to SNC + LNC (Fig 5), and to SNC + LNC + LC cells (Fig 6). Among these three, there was again a marginally significant difference for SNC + LNC cells ($P = .06$) (Fig 5); for the remaining two cell groups, the differences were not significant

Correlation of Cell Type and Cell Counts With Survival

Table 2 shows the survival analysis for various cell types on the basis of increasing cell counts. The P values listed are for pairs of survival curves at cell counts above and below sequential cut-off points. For LNC, the lowest P value occurred at a cut-off point of four cells; the P values then increased beyond the significant range. Based on these results, we could divide the patient population into only two groups ($P = 0.01$) (Fig 7).

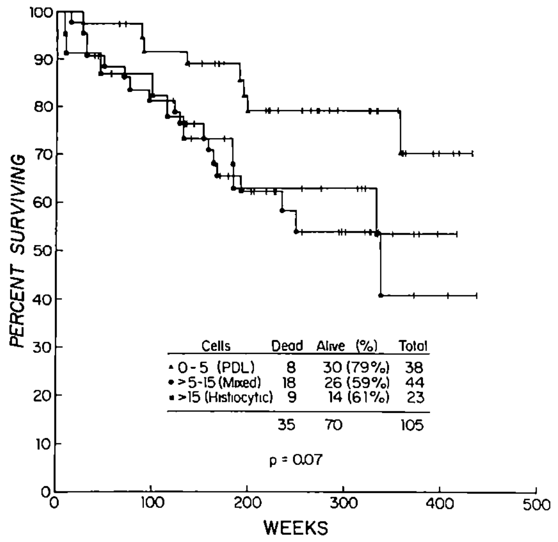

Fig 3. There were significant differences in survival times ($P = .07$) among the morphologic subtypes of follicular lymphomas when the standard Berard criteria (applied criteria to large noncleaved cells only) were used for subclassification of follicular lymphomas.

For SNC, the P values varied, with an initial lowest P value at a cut-off point of 3 cells; the P values then rose and began lowering again to the .01 range at 7 cells and attaining their lowest level at 10 cells and then rising once more. Based on these results, we divided the patient population into three groups (0–3 cells, >3 to 10 cells, and >10 cells), and a statistical comparison of the survival curves for these three groups showed significant differences ($P = .009$) (Fig 8).

For LNC + SNC cells, the analysis illustrated in Table 2 suggested that there might be three patient groups, with a clear break at seven cells and possibly another at 16 cells. Although the difference among the groups was significant ($P = .015$) (Fig 9), it was due solely to the superior survival of the first group (0 to 7 cells), with no separation between the latter two groups (7 to 16 cells and >16 cells) (Fig 9).

The analysis for all large cells (LNC + SNC + LC) suggested that there were three patient groups (0 to 8 cells, >8 to 13 cells, and >13 cells) (Table 2). Statistical compari-

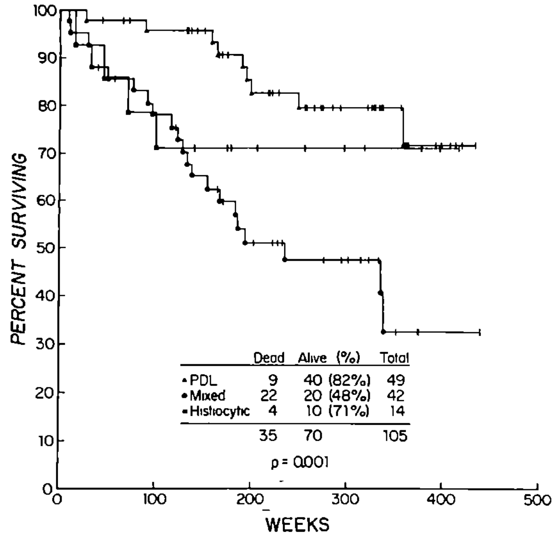

Fig 2. There were significant differences in survival times among the morphologic subtypes of follicular lymphomas when the SWOG diagnoses (subjective) were used ($P = .001$).

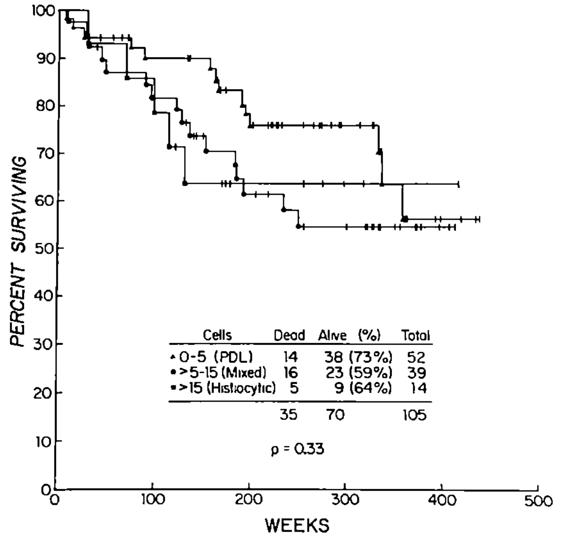

Fig 4. There were no significant differences in survival times ($P = .33$) among the morphologic subtypes of follicular lymphomas when the Berard method was modified (criteria applied to SNC).

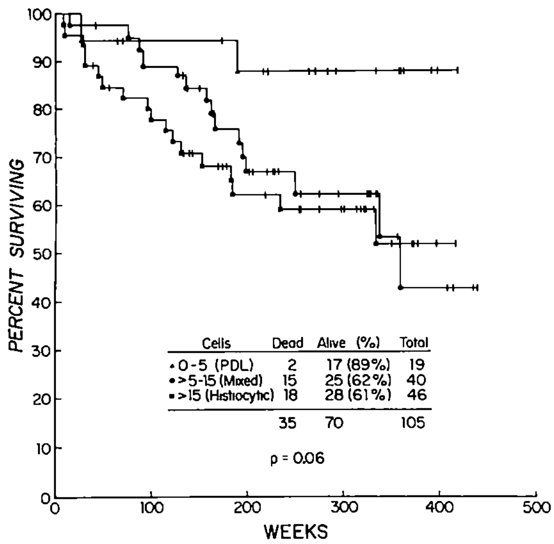

Fig 5. There were differences in survival times ($P = .06$) among the morphologic subtypes of follicular lymphomas when the Berard method was modified and applied to SNC plus LNC.

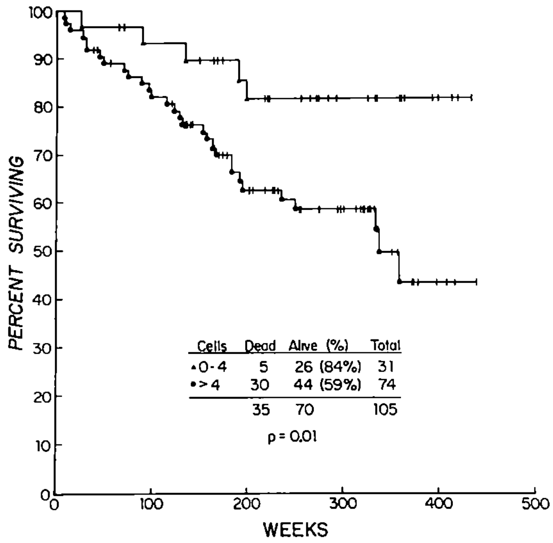

Fig 7. Patients with four or fewer LNC had survival times significantly superior to those of patients with more than four LNC cells ($P = .01$).

son of these three patient groups revealed a significant difference among the groups, with that of 0 to 8 cells having the longest survival ($P = .02$) (Fig 10). A pairwise comparison between the group with >8 to 13 cells and the group with more >13 cells yielded a P value of only 0.40, however.

DISCUSSION

Parameters Influencing Survival

This attempt to establish definite and objective criteria for the subdivision of FLs was undertaken on a group of 105 patients, all of whom uniformly received aggressive combination chemotherapy[17,18] regardless of the histologic type. To ascertain whether histologic variables independently influence survival, we performed multivariate Cox regression analyses, taking into consideration clinical parameters, diagnosis, and cell counts (Table 1). The results of this analysis showed that the stage of the lymphoma and patient age were the most important parameters. In addition, when the diagnosis by each of the pathologists as well as that of the SWOG and the consensus diagnosis were used, it was clear that histologic characteristics were an independent parameter influencing survival. Specifically, we found that, for all except one pathologist, either the diagnosis or the cell counts were independent parameters influencing survival. Thus, based on these analyses, we considered it worthwhile to

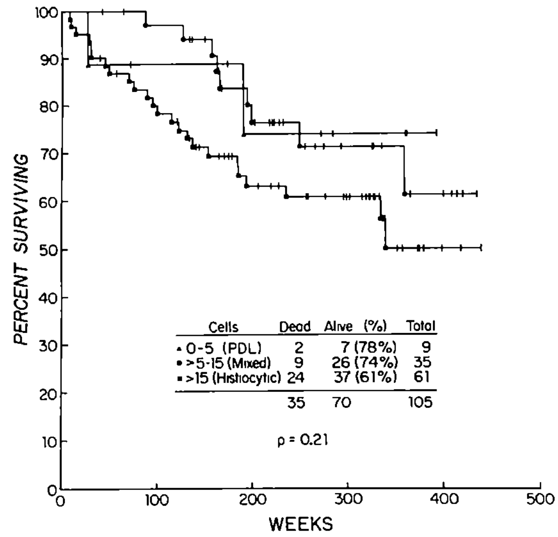

Fig 6. There were no differences in survival times ($P = .21$) among the morphologic subtypes of follicular lymphomas when the Berard method was modified and applied to all "large" cells (small noncleaved plus large noncleaved plus large cleaved cells [SNC + LNC + LC]).

Table 2. Paired Survival Analysis According to Objective Counts with Successive Cutoff Points (in Terms of Mantel-Cox P values)

Cutoff Point* (No of Cells)	LNC	SNC	LNC + SNC	LNC + SNC + LC
2	.149	.073	.172	.442
3	.038	$\boxed{014}$	.127	.172
4	$\boxed{014}$	037	.167	.306
5	026	.141	.037	.450
6	122	065	.008	.089
7	384	016	$\boxed{.004}$	.050
8	.700	.062	.011	$\boxed{.009}$
9	619	012	.025	.011
10	844	$\boxed{.010}$	.018	.026
11	.809	.047	033	.052
12	.674	.071	102	.019
13	.534	.097	140	$\boxed{.016}$
14	.971	.235	.258	.094
15	580	.571	.131	.080
16	.580	.406	$\boxed{.099}$	.250
17	976	.711	.099	.157
18	.777	.264	.176	.157

*Groups formed with cutoff point in lower group (eg, 0-2 $v > 2$).

Abbreviations: LNC, large noncleaved; SNC, small noncleaved, LC, large cleaved; ☐ designates the cutoff points (see also Table 3).

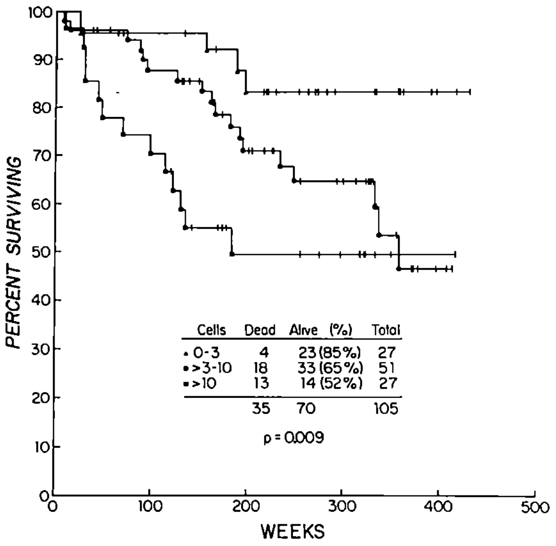

Fig 8. The patient population can be subdivided into three prognostic groups (*P* = .009). Patients with three or fewer SNC had the longest survival times, and patients with more than ten SNC cells had the shortest survival times. Patients with more than three and up to ten SNC cells had an intermediate prognosis.

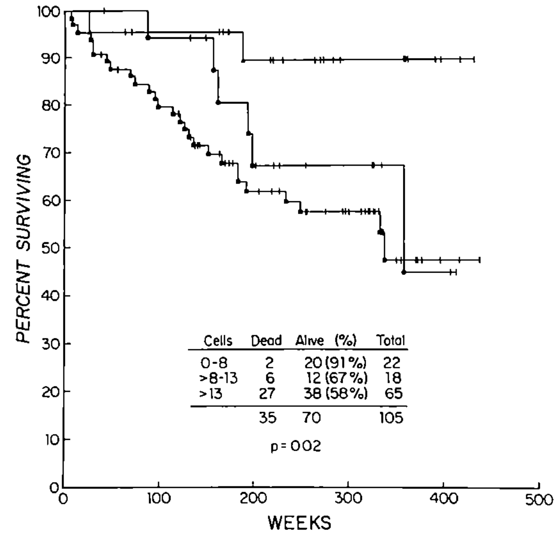

Fig 10. The patient population can be subdivided into two prognostic subgroups (*P* = .02). Patients with eight or fewer large cells (SNC plus LNC plus LC) had survival times significantly superior to those of patients with more than eight large cells.

investigate the influence of subjective diagnosis and actual cell counts with survival.

Survival Based on Subjective Diagnosis

Because one of our purposes in this study was to find out whether the subjective diagnosis[1,2,14] or actual counting of cells[15] (according to the standard and modified Berard methods) yields a meaningful separation of the survival curves, we first looked at the survival curves based on the subjective diagnosis.

When subjective diagnoses were rendered according to the Rappaport classification[2] (Fig 1), Lukes and Collins[14] classification, and the Working Formulation,[1] based on the consensus diagnosis, there were no significant differences in the survival curves among the different subtypes. This result is in contrast with published reports showing that the FLs could be separated into meaningful prognostic subgroups.[2-10] In most previous studies,[2-10,25,26] FLs have generally been treated non-aggressively. It has been possible to separate the patient populations studied into at least two, if not three, prognostic groups. In the last 5 years, however, the histiocytic FLs have been treated aggressively, and a significant percentage of the patients have achieved a prolonged disease-free survival, which suggests a cure.[9,10]

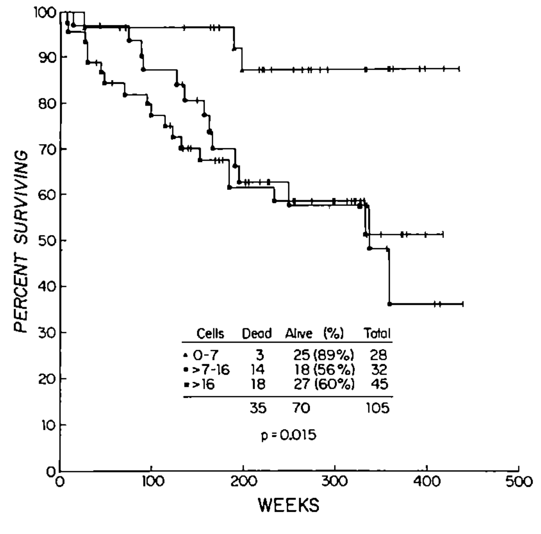

Fig 9. The patient population can be subdivided into two prognostic subgroups (*P* = .015). Patients with seven or fewer SNC plus LNC had survival times that were significantly superior to those of patients with more than seven SNC plus LNC cells.

For our 105 patients, the SWOG diagnosis of record showed that there were significant differences in survival times among the three subtypes of FL (Fig 2). Thus, in the standard process of arriving at the SWOG diagnoses of record, it appeared that histologic subclassification provided useful information; but this was not the case when the consensus diagnosis established in this study was used. Thus, in this study, even though some of the pathologists were able to make diagnoses that were predictive of survival, the consensus process used obscured these differences.

Survival Based on Actual Cell Counts

Use of standard Berard criteria for cut-off points. We used the cut-off points for LNC cells, as proposed by Berard, to ascertain whether this cell type was useful for the definition of prognostic subgroups. In this way, the patient population could be divided into two rather than three prognostic subgroups (Fig 3) (*P* = .07).

Use of the modified Berard method. We used the same cut-off points suggested by Berard also for SNC, LNC + SNC, LNC + SNC + LC cells, because (a) in the Lukes and Collins Classification and in the Working Formulation, SNC is an important prognostic cell type; (b) it may be difficult to distinguish the LNC and SNC cells from one another; and (c) in the Rappaport system, the so-called "large" cells include the combination of the three cell types (LNC, SNC, LC). Although Figs 4 and 6 do not show any significant separation of survival curves, Fig 5 shows that, by using the cut-off points of Berard for combined SNC + LNC cells, one could obtain results ($P = .06$) similar to those noted for LNC alone (Fig 3; see previous paragraph). These data give credence to the Berard definition of LNC and to the cut-off points that they proposed.[15]

Derivation of new cut-off points. When we applied our data to the generation of survival curves by using sequential cut-off points (Table 2), we were able to obtain significant separations, not only for LNC (Fig 7), but also for SNC (Fig 8), LNC + SNC (Fig 9), and LNC + SNC + LC cells (Fig 10). We were able to derive three distinct prognostic subgroups only for the SNC counts, however (Fig 8). For each of the others, we were able to obtain only two prognostic subgroups. It becomes apparent that, by using cell counts, one can separate the patient population into at least two groups for each of the cell types. This clearly indicates the importance of counting (Figs 7–10). In contrast, when we used the subjective consensus diagnosis without counting (Fig 1), we found no separation of survival curves.

These results clearly show that, when cut-off points different from Berard's are used, both SNC and LNC cells are prognostically important. Thus, in the future, counting of cells may lead to a significant and more reproducible subclassification of FLs. This finding raises an important issue· What are the criteria for distinguishing SNC cells from LNC cells? Berard has stated that he refers to cells as LNC only when he can readily identify multiple, distinct nucleoli. The fact that different pathologists could identify either LNC or SNC cells, but not both, as having prognostic significance suggests that the criteria used in this study for the identification of these two cell types were not uniformly applied, difficult to apply, or invalid.

The criteria for distinguishing these two cell types are indeed difficult to apply. For example, the nucleoli of LNC cells are supposed to be larger than the nucleoli of SNC cells, according to the published criteria of Lukes and Collins. In actual practice, however, the nucleoli of the LNC cell appears to be relatively smaller because the nuclear and cell size are larger. On the other hand, in the SNC cell, since the nuclear and cell size are smaller, the nucleoli appear to be relatively larger. Moreover, if the LNC cell has a single nucleolus, it may be confused with a benign histiocyte.

Treatment Considerations Based on Morphology

Past studies have shown that there are significant differences in the natural history, response to therapy, and patient survival among the different subtypes of FLs. It is well known that patients with NPDL lymphoma may not require any treatment initially.[25] When treatment is desired for relief of symptoms, there is no proven advantage of combination chemotherapy over the use of a single alkylating agent.[26] On the other hand, the nodular "histiocytic" lymphomas are thought to be curable and are, therefore, treated intensively.[9,10] Our findings cast doubt on some of these generally accepted treatment practices and create a dilemma for both practicing oncologists and researchers.

The results of our study cause concern for the following reasons:

1. Because the choice of treatment for the different subtypes of FLs is totally dependent on the histiologic diagnosis, it is imperative that the diagnosis rendered by the pathologist be reproducible and reliable. In our previous study, however, the diagnoses of seven "expert" pathologists had great variability, particularly when they were based on subjective evaluation, and major disagreement existed in 37% of the cases.[19] A major disagreement was defined as two extreme diagnoses made on the same case (eg, one or more of the pathologists diagnosed NPDL and one or more of the other pathologists in the group diagnosed NH). These data suggest that treatment decisions based on histologic criteria are sometimes misdirected.

2. Moreover, this study showed that when we used the *consensus* subjective diagnosis, we found no significant differences in survival among patients with the different subtypes of FLs, indicating that survival is not necessarily predicted by the histologic findings as perceived by a consensus of "experts."

3. The results of our previous study showed that the greatest disagreement among the pathologists concerned the NM category.[19] All seven pathologists could agree with the diagnosis of NM in only one of the 39 cases studied, and some of these were classified as NPDL, whereas others were categorized as NH.

These findings suggest that some patients with NPDL lymphoma may have a potentially curable form and that new techniques are needed for their identification. Routine administration of intensive drug regimens to all patients who have presumed NPDL lymphoma seems unreasonable, however, because most patients would experience unnecessary toxic effects.[26]

Debates regarding the appropriate management of NM lymphoma patients are moot because this disease category cannot be identified reproducibly. At institutions that have identified a single pathologist who has an established record of relating diagnoses to survival, treatment should be individualized. For most practicing oncologists, our results suggest that a diagnosis of NM lymphoma carries a substantial risk of being changed to NH lymphoma if a histologic review is conducted; consequently, patients should probably be given a trial of intensive chemotherapy designed for cure. In addition, investigators should be cautious to include the NM category in studies of either indolent or aggressive histologic types because the survival of these patients is not reproducibly predictable. As a possible solution to the problem, some investigators[27] have suggested that immunologic methods may be useful in the identification of subgroups of NM. Immunologic interpretations are often subjective, however, and very little information is available regarding the reproducibility and the interobserver agreement on the interpretation when the results are based on immunologic techniques.[28] Moreover, NM is a morphologic and not an immunologic diagnosis. It would be worthwhile to ascertain whether growth fractions as determined by immunostaining with monoclonal antibody Ki-67, which distinguishes low-grade from high-grade lymphomas,[29] is helpful in the subclassification of FL.

Some patients with a diagnosis of NH lymphoma are probably not curable and are likely to have an indolent disease. Nevertheless, a high percentage of patients with this diagnosis have aggressive disease and, like those with the NM type, should receive a trial of intensive chemotherapy.[9,10]

Can We Propose New Criteria (Cut-Off Points) for the Subclassification of FLs?

Based on the analyses illustrated in Table 2 and in Figs 7 through 10, we were able to generate cut-off points that might be useful for the subclassification of FLs (Table 3). These cut-off points would have to be verified independently on a different group of patients, however, and it would be necessary to ascertain whether they are indeed useful before they could be recommended for routine application.

Table 3. Suggested Optimal Cutoff Points for Prognostic Survival Groups

Cell Type(s)	Suggested Groupings		(No of Cells)
LNC	0–4	>4	
SNC	0–3	>3–10	>10
LNC + SNC	0–7	>7	
LNC + SNC + LC	0–8	>8	

Abbreviations: LNC, large noncleaved; SNC, small noncleaved; LC, large cleaved.

Conclusions

Even though our previous study[19] showed great variability among pathologists, certain conclusions can be drawn regarding the most appropriate method for subclassifying FLs. This could only be achieved because we analyzed the data in various ways (making a subjective diagnosis based on estimation of numbers of cells, counting cells and applying the standard and modified Berard methods to derive a diagnosis, and finally using data from these analyses to generate a new set of survival curves). We conclude the following:

1. Actual counting of cells is probably a method for arriving at a diagnosis that is superior to making a diagnosis based on subjective assessment of the percentages of different cells.
2. The cut-off points suggested by Berard for the subclassification of FLs based on the number of LNC cells appeared to be generally valid. This method divided the patients into only two groups with significantly different survival times In the future, however, this method may be more reproducible and may also make it possible to select the group that requires aggressive therapy.
3. The cells that probably have a major influence on survival are those transformed lymphoid cells which contain multiple, distinct nucleoli. These cells were presumably identified by some investigators as SNC cells and by others as LNC cells.
4. Whether large transformed lymphoid cells with relatively small nucleoli (LNC) have a different biologic behavior than do transformed cells of intermediate size with multiple, relatively large nucleoli (SNC) cannot be established from our study.
5. It is difficult for "expert" hematopathologists to separate favorable from unfavorable FLs reproducibly by using subjective morphologic criteria alone.
6. If patients with NM lymphoma are identified by pathologists who do not have an established record for predicting survival based on morphologic subclassification, these patients should receive intensive combination chemotherapy designed for cure.
7. More objective and reproducible methods are needed for the separation of patients into favorable and unfavorable prognostic groups.

ACKNOWLEDGMENT

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REFERENCES

1. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphoma: Summary and description of a working formulation for clinical usage. The non-Hodgkin's lymphoma pathologic classification project. Cancer 49:2112, 1982

2. Rappaport H: Tumors of the hematopoietic system, in Atlas of Tumor Pathology, Section III, Fascicle 8 Washington, DC, Armed Forces Institute of Pathology, 1966, pp 97–161

3. Jones SE, Fuks Z, Bull M, Kadin ME, Dorfman RF, Kaplan HS, Rosenberg SA, Kim H. Non-Hodgkin's lymphomas IV Clinicopathologic correlation in 405 cases. Cancer 31:806, 1973

4. Schein PS, Chabner BA, Canellos GP, Young RC, Berard C, DeVita VT. Potential for prolonged disease-free survival following combination chemotherapy of non-Hodgkin's lymphoma. Blood 43:181, 1974

5. Bloomfield CD, Goldman A, Dick F, Brunning RD, Kennedy BJ: Multivariate analysis of prognostic factors in the non-Hodgkin's malignant lymphomas. Cancer 33:879, 1974

6. Ezdinli EZ, Costello W, Wasser LP, Lenhard RE, Berard CW, Hartsock R, Bennett JM, Carbone PP. Eastern Cooperative Group experience with the Rappaport classification of non-Hodgkin's lymphomas Cancer 43:544, 1979

7. Rosenberg SA: Non-Hodgkin's lymphoma: Selection of treatment on the basis of histologic type. N Engl J Med 301:924, 1979

8. Glick JH: Chemotherapy of Hodgkin's lymphomas, in Bennett JM (ed): Lymphomas I Including Hodgkin's Disease. Boston, Martinus Nijhoff, 1981, pp 343–446

9. Osborne CK, Norton L, Young RC, Garvin AJ, Simon RM, Berard CW, Hubbard S, DeVita VT Jr: Nodular histiocytic lymphoma: An aggressive nodular lymphoma with potential for prolonged disease-free survival. Blood 56:98, 1980

10. Glick JH, McFadden E, Costello W, Ezdinli E, Berard CW, Bennett JM. Nodular histiocytic lymphoma: Factors influencing prognosis and implications for aggressive chemotherapy. Cancer 49:840, 1982

11. Butler JJ, Stryker JA, Schullenberger CC. A clinicopathologic study of stages I and II non-Hodgkin's lymphomata using the Lukes-Collins classification. Br J Cancer 31:208, 1974 (suppl II)

12. Anderson T, Bender RA, Fisher RI, DeVita VT, Chabner

BA, Berard CW, Norton L, Young RC: Combination chemotherapy in non-Hodgkin's lymphoma: Results of long-term follow-up. Cancer Treat Rep 61.1057, 1977

13. Glick JH, Barnes JM, Ezdinli EZ, Berard CW, Orlow EL, Bennett JM: Nodular mixed lymphoma: Results of a randomized trial failing to confirm prolonged disease-free survival with COPP chemotherapy. Blood 58:920, 1981

14. Lukes RJ, Collins RD: Immunologic characterization of human malignant lymphomas. Cancer 34:1488, 1974

15 Mann RB, Berard CW. Criteria for the cytologic subclassification of follicular lymphomas A proposed alternative method. Hematol Oncol 1:187, 1983

16 Hoppe RT. A working formulation of non-Hodgkin's lymphomas for clinical usage: Clinicopathological and prognostic correlations, in Rosenberg SA, Kaplan HS (eds). Malignant Lymphomas. Orlando, Fla, Academic Press, 1982, pp 469–483

17. Jones SE, Grozea PN, Metz EN, Haut A, Stephens RL, Morrison FS, Talley R, Butler JJ, Byrne GE Jr, Hartsock R, Dixon D, Salmon SE· Improved complete remission rates and survival for patients with large cell lymphoma treated with chemoimmunotherapy: A Southwest Oncology Group study. Cancer 51:1083, 1983

18. Jones SE, Grozea PN, Miller TP, Van Slyke EJ, Balcerzak SP, Costanzi JJ, Morrison FS, Eyre HJ, Fabian CJ, Dabich L, Dixon DO, Hartsock RJ, Grogan TM, Kjeldsberg C, Schnitzer B· Chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone alone or with levamisole or with levamisole plus BGC for malignant lymphoma: A Southwest Oncology Group study J Clin Oncol 3·1318, 1985

19. Metter GE, Nathwani BN, Burke JS, Winberg CD, Mann RB, Barcos M, Kjeldsberg CR, Whitcomb CC, Dixon DO, Miller TP, Jones SE. Morphological subclassification of follicular lymphoma: Variability of diagnoses among hematopathologists, a collaborative study between the Repository Center and Pathology Panel for Lymphoma Clinical Studies. J Clin Oncol 3:25, 1985

20. DeVita VT, Rappaport H, Frei E III: Announcement of formation of The Lymphoma Task Force and Pathology Reference Center Cancer 2:1087, 1968

21. Kim H, Zelman RJ, Fox MA, Bennett JM, Berard CW, Butler JJ, Byrne GE, Dorfman RF, Hartsock RJ, Lukes RJ, Mann RB, Neiman RS, Rebuck JW, Sheehan WW, Variakojis D, Wilson JF, Rappaport H: Pathology Panel for Lymphoma Clinical Studies· A comprehensive analysis of cases accumulated since its inception. JNCI 68.43, 1982

22. Kaplan EL, Meier P Nonparametric estimation from incomplete observations. J Am Stat Assoc 53 457, 1958

23. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163, 1966

24. Cox DR: Regression models and life tables. J R Stat Soc 34:187, 1972

25 Portlock CS, Rosenberg SA: No initial therapy for stage III and IV non-Hodgkin's lymphomas of favorable histologic types. Ann Intern Med 90.10, 1979

26 Rosenberg SA: The low-grade non-Hodgkin's lymphomas: Challenges and opportunities J Clin Oncol 3.299, 1985

27 Grogan TM, Hicks MH, Jolley CS, Rangel CS, Jones SE. Identification of two major B-cell forms of nodular mixed lymphoma Lab Invest 51:504, 1984

28. Warnke RA, Rouse RV: Limitations encountered in the application of tissue section immunodiagnosis to the study of lymphomas and related disorders Hum Pathol 16:326, 1985

29. Gerdes J, Dallenback F, Lennert K, Lemke H, Stein H: Growth fractions in malignant non-Hodgkin's lymphomas (NHL) as determined in situ with the monoclonal antibody Ki-67 Hematol Oncol 2:365, 1984

Leu 11$^+$ Tγ Cell Chronic Lymphocytic Leukemia With Partially Activated Natural Killer Function and Its Further Activation by Recombinant IL2 In Vitro

By Shinichi Tagawa, Yukihiro Tokumine, Etsuko Ueda, Koji Waki, Yoshio Kanayama, Nobuhiro Taniguchi, Toshihiro Nakanishi, Ryoichi Inoue, and Teruo Kitani

A patient with Tγ cell chronic lymphocytic leukemia with the Leu 11$^+$ phenotype and novel function of activated natural killer cells is reported. The peripheral blood mononuclear cells of this patient showed large granular lymphocytes by May-Giemsa staining and lamellipodia by scanning electron micrography. Tests on reactivity with monoclonal antibodies showed that most cells were Leu 11$^+$, OKT3$^-$/Leu 1$^-$, OKT4$^-$, OKT8$^-$, Leu 7$^-$, OKM1$^-$, and Tac$^-$. Freshly collected cells lysed not only K562, which is highly sensitive to natural killer cells, but also Raji cells and Daudi cells, which are not. Leu 11$^+$ cells were triggered by recombinant interleukin 2 (rIL2) to proliferate, produce γ-interferon (γIFN), and show enhanced HLA-DR antigen expression, and 30% of the Leu 11$^+$ cells became positive for IL2 receptor antigen (Tac). The spectrum of cytotoxic activity of these cells against target cells was extended by rIL2; after treatment with rIL2, the cells also lysed HeLa cells and even fresh cancer cells. This stimulation also increased the activities of acid phosphatase and tartrate-resistant acid phosphatase of the cells and resulted in the appearance of nonspecific esterase activity. The expanded cell population may represent a neoplasm, but these findings provide information on a novel differentiation stage of activated NK cells.

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CHRONIC lymphocytic leukemia (CLL) involving Tγ cells with the morphology of large granular lymphocytes (LGL) has been described by many investigators[1-6] and reviewed by Reynolds and Foon.[7] Patients with this disease have persistently elevated numbers of Tγ cells, but their clinical courses are less severe than in other T cell malignancies, and often anemia or neutropenia is the only symptom.

In healthy persons, the Tγ cell population consists almost entirely of natural killer (NK) cells, which have the morphologic characteristics of LGL,[8-11] produce lymphokines,[12-14] and regulate hematopoiesis.[15] Thus, study of patients with Tγ-CLL with NK activity should provide information on the origin of NK cells and their spectrum of lysis of specific targets, lymphokine production, and stage of differentiation.

Here we report detailed studies on cells of a patient with T cell leukemia of the Leu 11$^+$ phenotype showing novel activated NK functions. Leukemic cells expressed the Leu 11 marker, an antigen associated with the Fc-IgG receptor of human NK cell.[16] The cells of this patient mediated both strong antibody-dependent cell-mediated cytotoxicity (ADCC) and NK activity with an unusual specificity spectrum; freshly collected cells lysed not only K562, which is highly sensitive to NK cells, but also Raji and Daudi cells, which are relatively NK-insensitive B cell lines. Thus, the cells had the cytolytic specificity spectrum of the activated NK cells,[17-20] but this spectrum was different from that of lymphokine-activated killer (LAK) cells,[21] because freshly collected cells did not lyse cultivated human cancer cells, HeLa cells, or fresh cancer cells. Thus, we called this cytolytic activity partially activated NK function. The activated NK features of leukemic cells of this patient suggest a novel differentiation stage of human NK cells.

MATERIALS AND METHODS

Case report. A 65-year-old man first noticed fatigue and malaise following exercise in March 1983. In June, 1983, his hemoglobin was 5 0 g/dL, his WBC count was 11,400 cells/μL, his platelet count was 19 × 10^4/μL, and his lymphocyte count was 10,260 cells/μL. Physical examination in the same month revealed no abnormalities, liver and spleen were normal size, there was neither lymphadenopathy nor skin lesions. The delayed cutaneous hypersensitivity reaction to a purified protein derivative of tuberculin was positive. Serum lactic dehydrogenases was within the normal range The serum Ig levels were 132 mg of IgM/dL, 1,240 mg of IgG/dL, and 289 mg of IgA/dL. Bone marrow aspiration was done; leukemic cells were found to constitute 60% of the nuclear elements Informed consent was obtained prior to this study. A diagnosis of CLL was made

In July 1983, the patient was transferred to the Hospital of the Research Institute for Microbial Diseases at Osaka University. His serum was negative for antibodies to adult T cell leukemia-associated antigens (ATLA). The hematologic profile on July 22, 1983 is shown in Table 1. Leukopheresis of 1 × 10^8 cells was done once every 2 weeks with transfusion of 400 mL of blood; these procedures have been continued and, at present (February 1986), the condition of the patient is under control, with a WBC count of 13,300 cells/μL, a platelet count of 9.4 × 10^4/μL, and a lymphocyte count of 12,635 cells/μL. Chromosomal study showed that the patient's karyotype was normal.

Detection of ATLA antibodies. A test for detection of ATLA antibodies[22] in the patient's serum was done by enzyme immunoassay with nitrocellulose membrane strips blotted with partially purified ATLA-related proteins from an ATLV/HTLV producing cell line, MT-2[23] (kindly provided by Dr I Miyoshi, Kotch Medical School, Japan).

Cells and cell fractionation. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Paque density-gradient centrifugation. The cells were further separated into a fraction without cells positive for Leu 1 (Leu 1-depleted fraction) and a fraction without leukemic cells (Leu 11-depleted fraction) by complement-mediated lysis with the respective monoclonal antibodies. The extent of depletion of the respective cells was checked by indirect immunofluorescense. Leu 11$^+$ cells were separated in a FACS IV cell sorter (Becton Dickinson, Sunnyvale, Calif) for scanning electron microscopic examination.

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Table 1. Hematologic Profile

Parameter	Patient	Normal
Red blood cells		
Hemoglobin	8.3*	13.5–17.0*
Platelets	26.2×10^4†	13.5×10^4–37.5×10^4†
White blood cells		
Types of leukocytes		
Neutrophils	651 (3)	1,500–6,000 (38–71)
Lymphocytes	20,941 (96.5)	1,000–4,000 (26–46)
Monocytes	109 (0.5)	0–500 (0–6)
Total	21,700	4,000–8,400
Lymphocytes		
Total	20,941	1,000–4,000
T cells	20,313 (97)	(74.3 ± 5.6)
Tγ	16,962 (81)	(27.2 ± 5.6)
B cells	105 (0.5)	(12.1 ± 5.0)
Null cells	524 (2.5)	

Numbers in parentheses are percentages.

*g/dL.

†Absolute number.

Surface markers. Test of formations of E rosettes and EAC rosettes and for the presence of Fcγ receptor (EAγ) and surface immunoglobulin were performed as described previously.[24] Examination of the stainings of the cells with the monoclonal antibodies OKT3, OKT4, OKT6, OKT8, OKM1, OKIa1, OKT11, Leu 1, Leu 7, Leu 10, Leu 11b, and anti-Tac[25] (kindly provided by Dr T. Uchiyama, Kyoto, Japan) was done by the indirect immunofluorescence test. FITC-conjugated monoclonal antibody, Leu 11a, was used for double staining of cells with Leu 11 and anti-Tac.

Tests of cytolytic activity. NK activity was measured at effector–target cell (E–T) ratios of 30:1 and 15:1, in a 4-hour ^{51}Cr release assay.[5] For examination of ADCC, ^{51}Cr-labeled sheep erythrocytes sensitized with rabbit antibody (Ig G fraction) were used as targets in a 4-hour test, at E–T ratios of 1:1 and 2:1. For LAK assays,[21] cells were cultured at 2×10^6/mL with 400 U/mL of rIL2 for 3 days. The target cells used were K562, Raji cells, Daudi cells, HeLa cells, and cancer cells from the ascites of a patient with ovarian cancer. Because these cancer cells were in clusters, they were separated into single cells by trypsinization.[21]

IL2 response. Comparison of the relative activities of rIL2 and standard natural IL2 (500 U/mL: Jurkat) provided under the NCI Biological Response Modifier (BRM) Program by measurement of the proliferative responses to IL2 of a mouse cell line (NKC3) showed that 1 U of rIL2 is equivalent to 100 U of standard IL2.[26] Thus, units of rIL2 are here expressed in terms of units of the standard.

The responses of the cells to rIL2 were examined essentially as described previously.[24] In brief, samples of 3×10^5 normal PBMCs or PBMCs from the patient were suspended in 150 mL of serum-free medium. The suspensions were incubated in the presence of an appropriate concentration of rIL2, and pulsed with [^{3}H]thymidine 6 hours before harvest. The stimulation index (SI) is the ratio of [^{3}H]thymidine incorporation into stimulated and control cultures.

Interferon (IFN) assay. Mononuclear cells were suspended at 2×10^6 cells/mL in serum-free medium,[27] and incubated at 37 °C for 3 days with 400 U/mL of rIL2. IFN was assayed by measuring inhibition of the cytopathic effect of Shindbis virus of FL amnion cells. The type of IFN produced by the lymphocytes was determined by neutralization tests with anti-αIFN, anti-γIFN (Interferon Science, New Brunswick, NJ), anti-βIFN (Japan Immunomonitoring Center Inc, Tokyo, Japan), and acid lability test.[28]

Agarose–single-cell cytotoxicity assay. Single-cell cytotoxicity assay was performed essentially as described.[29,30] In brief, mixtures of equal numbers of effector and target cells were centrifuged, incubated, and then gently resuspended in 1% agarose in RPMI 1640 at 40 °C. The mixture was immediately plated in a Petri dish. When the agarose had hardened, it was overlaid with RPMI 1640 containing 5% fetal calf serum (FCS). After 3-hour incubation, the medium was removed, and the plates were stained with trypan blue, fixed with 1% formaldehyde, and examined with an inverted microscope.

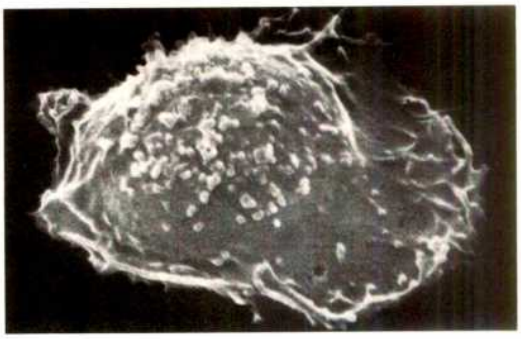

Fig 1. Scanning electron micrograph of leukemic cell. Rug-like extensions (lamellipodia) are attached to the surface. (Original magnification, ×7,000; current magnification ×4,550.)

The proportion of effector cells bound to target cells was scored as the percentage of conjugate-forming cells (%CFCs). The percentage of dead targets in conjugates was determined by counting the number of dead target cells in 100 conjugates. The background count of spontaneous cell death was determined by counting the number of dead target cells in control dishes. The percentage of lytic conjugate-forming cells (%LCFCs) was calculated as: the percentage of dead targets in conjugates minus the percentage of spontaneously dead targets × the percentage of dead targets in conjugates per 100. The percentage of active killer cells (%AKC) was calculated as %CFCs × %LCFCs/100.

RESULTS

Morphology and cytochemistry. The cells were large (12.53 μm across), with a high ratio of cytoplasm to nucleus, and azurophilic granules in the cytoplasm. On cytochemical analysis, acid phosphatase in the cells was seen as scattered positive granules throughout the cytoplasm. The cells were weakly positive for tartrate-resistant acid phosphatase

Table 2. Cell Surface Antigen Phenotype of Peripheral Blood Mononuclear Cells from Patient

	Cells With Surface Antigens (%)	
Surface Marker	Patient	Normal*
E	97	74.5 ± 5.6
EAγ	81	27.3 ± 5.6
OKT3	11	67.7 ± 8.7
OKT4	8	44.7 ± 7.0
OKT8	10	24.1 ± 6.5
OKIa1	23	8.0 ± 4.1
OKT11	82	73.5 ± 5.3
OKM1	11	9.4 ± 7.2
Leu 1	12	71.0 ± 7.0
Leu 7	0	15.8 ± 3.9
Leu 11b	81	15.4 ± 3.4
Tac	0	5.6 ± 1.8

*Values are mean percentages of reactive cells ± SD (n = 10).

Table 3. Changes in Expression of Surface Antigen of Patient's Cells After Stimulation With rIL2

Monoclonal Antibodies	Freshly Separated Cells	rIL2 (400 U/mL)	PHA (1 μg/mL) + rIL2 (400 U/mL)	γIFN (10^4 U/mL)
OKIa1 (HLA-DR)	23	82	94	28
Leu 10 (HLA-DC)	15	88	84	ND
TAC (IL2 receptor)	0	36	68	0
Leu 11 (NK cells)	82	63	70	ND
Tac/Leu 11		30	80	

rIL2, recombinant interleukin 2; PHA, phytohemagglutinin; γIFN, gamma interferon.

*Because the expression of Tac antigens were low when macrophages were depleted, peripheral blood mononuclear cells not depleted of macrophages were used.

(TRAP), but they contained no detectable nonspecific esterase. After stimulation with rIL2 (400 U/mL) for 2 days, their acid phosphatase activity increased, their TRAP became intensely positive, and nonspecific esterase became detectable.

Electron microscopic examination showed tubular structures in parallel tubular arrays in the cytoplasm, which are characteristic of Tγ cells.[31,32] Scanning electron micrography (Fig 1) showed lamellipodia[33] in the leukemia cells. These peculiar structures were also seen in ~10% of PBMCs of normal persons. Examination of the fraction of Leu 11$^+$ cells separated from PBMCs of a healthy subject showed that many of them (78%) had this structure. Thus, lamellipodia are commonly present on normal and leukemic Leu 11$^+$ cells.

Marker analysis of lymphocytes. Relevant results of surface marker studies of freshly separated cells from our patient are listed in Table 2. The leukemic cells were Leu 11$^+$ and OKT11$^+$, but OKT3$^-$. After stimulation with rIL2 in vitro, the percentage of cells expressing HLA-DR antigens increased from 23% to 82% (Table 3). At the same time, 30% of the Leu 11$^+$ cells became positive for Tac antigen, as shown by double staining. We next studied whether the enhanced expression of HLA-DR antigen was the direct effect of rIL2 or was mediated by γIFN. Results showed that addition of sufficient recombinant γIFN without IL2 did not enhance expression of HLA-DR antigen. Thus, the enhancement seemed to be due to a direct effect of rIL2.

Table 4. Response to rIL2

Subject/Parameter	Not Stimulated	Stimulated With rIL2 (400 U/mL)
Patient: PBMCs (3×10^5 cells per well) cpm	11,679 ± 2,116*	84,687 ± 2,728
SI		7.3
Patient: Leu 1-depleted fraction (3×10^5 cells per well) cpm	4,030 ± 831	111,890 ± 3,672
SI		27.7
Normal controls: PBMCs (3×10^5 cells per well) cpm	4,080–5,919	18,045–31,988
SI		3.0–7.8

PBMCs, peripheral blood mononuclear cells; rIL2, recombinant interleukin 2.

The proliferative response to rIL2 was determined on day 3, 6 hours after addition of 1 μCi of [^{3}H]thymidine to cultures.

*Means ± SD for triplicate cultures.

Proliferation of the patient's lymphocytes in response to rIL2. The proliferative responses of cells to rIL2 are summarized in Table 4. The proliferation of the patient's cells without stimulation was twice that of cells from healthy individuals. After rIL2 stimulation, the SI of the patient's cells became 7.3 in 3 days. Moreover, the proliferative response of the Leu 1-depleted cells of the patient's cell fraction, most of which were Leu 11$^-$ cells, was much greater than that of PBMCs from normal persons. This finding suggested that Leu 11$^+$ cells respond to rIL2 (Table 4).

IFN production by the patient's lymphocytes in response to rIL2. Results on IFN production by PBMCs from the patient in response to rIL2 are shown in Table 5. IFN production by PBMCs from four healthy persons was also measured for comparison. The antiviral activity in the supernatant fluids appeared to be γIFN because it was inhibited at pH 2.0 and was inhibited completely by anti-γ IFN antiserum but was not affected by anti-α or anti-β IFN antiserum (Table 6).

Spectrum of specificity of the cytolytic activity of the patient's cells and its enlargement after stimulation with rIL2. Freshly collected cells from the patient lysed not only highly NK-sensitive target K562 cells, but also Raji and Daudi cells, which are not sensitive to NK (Table 7). After stimulation with rIL2 in vitro for 3 days, the cells were able to lyse HeLa cells and fresh cancer cells. The Leu 1-depleted fraction of the cells still showed this spectrum of lytic activity against these target cells, suggesting that Leu 11$^+$ leukemic cells are responsible for this activity. The cytotoxic activities

Table 5. IFN Production by Cells from Patient After Stimulation With rIL2

Donor	IFN (U/mL)	
	3 Days	6 Days
Patient	607	607
Normal controls (n = 4) mean ± SE	270.25 ± 126.15	472.75 ± 251.55

In a preliminary study, interferon (IFN) production by cells from the patient reached a plateau when >100 U/mL of recombinant interleukin 2 (rIL2) was added to 2×10^6 cells/mL. Therefore, IFN production on stimulation with 400 U/mL of rIL2 was measured.

Table 6. Characterization of IFN Produced by Cells From Patient After Stimulation with rIL2

Treatment	Residual IFN Activity (Percentage of Control)
—	100
Anti-α-IFN	100
Anti-β-IFN	100
Anti-γ-IFN	0
pH 2.0	<1

Abbreviations as in Table 5.

of cells collected at four subsequent times were also examined. Results indicated that the activated NK function and the appearance of LAK activity against HeLa cells on stimulation with rIL2 were reproducible (Table 8). In addition, freshly collected cells mediated ADCC against antibody-coated sheep erythrocytes. No phagocytosis of these erythrocytes by lymphocytes was observed, however.

Studies on cytotoxic activity at the single cell level. As shown in Table 9, >50% of the peripheral lymphocytes of the patient bound not only to the NK-sensitive target cells K562, but also to the NK-resistant target, Raji and Daudi cells. After incubation for 3 hours, most CFCs had lysed the target cells. In contrast, 10% to 14% of the peripheral lymphocytes from healthy donors bound to these targets, and 29.2%, 13.1%, and 8.4% of the CFCs lysed K562, Raji, and Daudi cells, respectively.

The %AKCs among cells of the patient varied little with different targets, being 39.8, 34.3, and 36.6 with K562, Raji, and Daudi cells, respectively. In contrast, the %AKCs in normal peripheral lymphocytes varied with different targets, the mean percentage with these targets being 2.2%, 1.8% and 1.1%, respectively. For detection of the proportion of Leu 11$^+$ cells in CFCs, lymphocytes of the patient were treated with FITC-conjugated Leu 11a, and the percentage of CFCs against each target in the resuspended materials without added agarose was determined in an immunofluorescence microscope. Most of the CFCs against each target (>98%) were Leu 11$^+$ cells. On stimulation with rIL2, the %CFCs and %AKCs of the leukemic cells against each target increased (data not shown).

Table 8. Reproducibility of Data on Cytotoxicity of Cells From Patient

Date of Sampling*	Percentage of Cytotoxicity of Fresh Cells			LAK (%)†
	K562	Raji	HeLa	HeLa
7/11/84	72.4	69.1	9.2	74.1
4/26/85	73.2	58.8	13.3	80.6
8/23/85	76.3	68.0	14.5	75.4
10/30/85	79.8	48.7	15.5	85.7

LAK, lymphokine-activated killer cells.

Cells were collected at four different times The E:T ratio was 30:1 in all experiments. Results are means for triplicate measurements SDs did not exceed 5%.

*Frozen cells were used in these experiments.

†For LAK assays, cells were cultured at 2 × 10^6/mL with 400 U/mL of rIL2 for 3 days.

DISCUSSION

Phenotypic studies of cases of Tγ lymphoproliferative diseases (Tγ-LPD) by Rambaldi and colleagues[34] showed that cells from at least 2 of 11 patients were Leu 11$^+$ (B73.1). Similarly, we found that 2 of 3 patients were Leu 11$^+$. Thus, it is likely that many cases of Tγ-LPD are Leu 11$^+$ Cells from most cases with the Leu 11$^+$ phenotype[5,34] were positive for OKT3, an antigen associated with the T cell antigen receptor. In contrast, cells from the present patient were Leu 11$^+$, but OKT3$^-$. An interesting finding was that the freshly isolated cells with the Leu 11$^+$ and OKT3$^-$ phenotype from this patient showed activated NK function. No leukemia cells with this function have been reported previously, and peripheral unstimulated NK cells from normal persons do not mediate this activated NK function.

In determining whether the cytotoxic spectrum characteristics of activated NK cells of this patient was attributable to the leukemic cells, which constituted most of the population of peripheral blood lymphocytes, or to the small proportion of residual normal lymphocytes, it was insufficient to consider only the overall cytotoxicity parameters. However, with an assay for detection of single cytotoxic NK cells, originally developed by Grimm and Bonavida,[29] the overall cytotoxicity could be separated into its component parts of effector cell frequency and target cell lysis per effector. The assay showed

Table 7. Specificity Spectrum of Cytotoxic Activity of Patient's Cells

Donor	Treatment of Effector Cells	Percentage of Cytotoxicy									
		Target K562		Target Raji Cell		Target. Daudi Cell		Target HeLa Cell		Target Ovarian Cancer	
		E.T.30:1	E.T 15.1	E.T.30 1	E T 15·1	E.T.30:1	E T 15 1	E T 30.1	E.T 15 1	E.T 30.1	E T 15.1
Patient	None	70.4	67.4	63.0	55.2	89.7	87.3	8.5	6.2	9.6	5.3
Patient	Cultured for 3 days with rIL2 (400 U/mL)	83.0	85.4	98.5	99.0	93.5	94.7	66.8	62.8	20.7	20.3
Patient	Cultured for 3 days with rIL2 (400 U/mL), then depleted of the Leu1$^+$-fraction	80.9	80.9	99.4	99.3	95.2	95.4	65.6	64.7	19.8	18.5
Normal donor	None	64.9	43.5	7.2	2.5	13.1	11.3	0.1	−1.2	1.0	0.05
Normal donor	Cultured for 3 days with rIL2 (400 U/mL)	58.3	51.8	52.6	24.3	91.8	63.5	98.0	68.6	17.4	14.8

Abbreviations as in Tables 4 and 5.

Results are means for triplicate determinations. SDs did not exceed 5% in each experiment. PBMCs from the patient depleted of macrophages and adherent cells were used as effector cells

Table 9. Capacity of Patient's Peripheral Lymphocytes to Bind to and Kill Targets

Source of Cells	Target Cells								
	K562 Cells			Raji Cells			Daudi Cells		
	%CFCs*	%LCFCs†	%AKCs‡	%CFCs	%LCFCs	%AKCs	%CFCs	%LCFCs	%AKCs
Patient§	52	76.5	39.8	57	60.1	34.3	53	69.1	36.6
Controls‖	10.3 ± 1.7	20.2 ± 8.5	2.2 ± 1.4	13.7 ± 1.9	13.1 ± 8.6	1.8 ± 1.3	11.7 ± 1.2	8.4 ± 4.1	1.1 ± 0.5

*%CFCs, percentage of conjugate-forming cells

†%LCFCs, percentage of lytic conjugate-forming cells

‡%AKCs, percentage of active killer cells

§Results are means for triplicate determinations in one of three experiments that gave similar results SDs were <5% of mean values

‖Mean percentages ± SD for three experiments on cells from different individuals

that AKCs in the cells of the patient exceeded 30%, whereas AKCs in cells from normal donors ranged from 1.1% to 2.2% with different targets. These findings support the hypothesis that the large population of Leu 11$^+$ cells among the leukemic cells of the patient, not the minor population of residual normal cells, mediated the activated NK function.

The lytic specificity spectrum of the cells of the patient is different from that of normal NK cells and the normal Leu 11$^+$ subset.[18,20] The culture of normal Leu 11$^+$ cells with K562[35] or IL2 in vitro can give rise to NK cells with this spectrum of lytic activity. Phillips and Lanier[35] designated NK cells with activated NK function as NK cells in a differentiation stage of NK cells. The Leu 11$^+$ leukemic cells of this patient may be due to clonal expansion of this novel differentiation stage of NK cells that has never been recognized in vivo before.

Four changes in parameters of the cells in response to rIL2 stimulation were observed. One was expansion of the cytotoxic spectrum of the leukemic cells. This phenomenon has not been reported previously. A second was increase in the activities of enzymes in azurophilic granules. A third was expression of HLA-DR antigens on the surface of the cells. In macrophages, γIFN is reported[36] to play a key role in the induction of HLA-DR antigens. In contrast, in the Leu 11$^+$ leukemic cells, we obtained evidence that the enhancement was due to a direct effect of rIL2. A fourth change was in Tac antigen expression. It is interesting that Tac antigens, which are now considered to be IL2 receptor molecules,[37] were not expressed on freshly collected cells, but were expressed de novo on Leu 11$^+$ cells in response to rIL2 in vitro.

There are at least two possible explanations for this finding. One is that the number of IL2 receptors expressed on one cell is too small to be readily detectable with anti-Tac monoclonal antibody by indirect immunofluorescence. Alternatively, cells may possess a distinct IL2 receptor that cannot react with antibody against the Tac epitope. The latter possibility is supported by reports that Tac-antigen negative cell subsets of normal peripheral lymphocytes such as LGL,[13] Leu 11$^+$ cells,[20] and T cells[38] respond to IL2 and that anti-Tac antibody does not inhibit the increase of NK activity induced by rIL2.[19,39]

Unlike those in other reports,[40] the cells of our patient produced γIFN. In contrast to the report of Hooks and colleagues,[2] γIFN production by the cells was not spontaneous, but was regulated by IL2. Zoumbus and co-workers[41] found that both α and γIFN are mediators of hematopoietic suppression in aplastic anemia. In patients with aplastic anemia, the IFN levels in the bone marrow are high, although circulating IFN is not always detected. Thus, in our patient, the γIFN produced by Leu 11$^+$ cells in the bone marrow may cause pancytopenia, although IFN was not detected in serum samples.

The cells of our patient had lamellipodia,[33] which were previously reported to be characteristic of activated T cells. The leukemic cells with the phenotype of Leu 11$^+$ and Leu 7$^+$, which we reported previously,[5] also had lamellipodia. These findings suggested that lamellipodia might be characteristic of Leu 11$^+$ cells. To investigate this possibility, we examined the morphology of normal Leu 11$^+$ cells obtained with a cell sorter. Scanning electron microscopy showed that ~10% of PBMCs had lamellipodia. Macrophages could be excluded in SEM studies, and most of the Leu 11$^+$ cells examined were found to have lamellipodia. Thus, we confirmed that the SEM feature of lamellipodia is commonly present on normal and leukemic Leu 11$^+$ cells (Inoue et al, manuscript in preparation).

Recently, DNA sequences coding for the β chain of the T cell receptor (Tβ) have been isolated.[42] Waldmann and co-workers[43] found that the patterns of Tβ gene rearrangement can be used to identify the lineage and clonality of human lymphoid neoplasms. Among patients with Tγ-LPD examined, T3-positive patients showed Tβ gene rearrangement,[34,43,44] whereas T3-negative patients did not.[34,44] Moreover, Lanier and co-workers observed that the Tβ gene was not rearranged in Leu 11$^+$ cells from normal PBMCs.[45] Thus, the cell lineage of T3-negative cases such as that of the present patient requires further characterization.

Our patient with Tγ-CLL had cells that showed many features of activated NK, and remained responsive to rIL2. The pathogenesis of this lymphocytic disorder is unknown. This study provides more precise information than that which could be obtained from normal heterogeneous NK cells on a maturation stage of activated NK cells that has not previously been recognized in vivo.

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REFERENCES

1 Bom-van Noorloos AA, Pegels HG, van Oers RHJ, Silberbusch J, Feltkamp-Vroom TM, Goudsmit R, Zeijlemarker WP, von dem Borne AEGK, Melief CJM: Proliferation of Tγ cells with killer-cell activity in two patients with neutropenia and recurrent infections. N Engl J Med 302:933, 1980

2. Hooks JJ, Haynes BF, Detric-Hooks B, Diehl LF, Gerrard TL, Fauci AS. Gamma (immune) interferon production by leukocytes from a patient with a Tγ cell proliferative disease. Blood 59 198, 1982

3. Itoh K, Tsuchikawa K, Awataguchi T, Shiiba K, Kumagai K· A case of chronic lymphocytic leukemia with properties characteristic of natural killer cells. Blood 61.940, 1983

4. Pandolfi F, De Rossi G, Semenzato G, Quinti I, Ranucci A, De Sanctis G, Lopez M, Gasparotto G, Aiuti F: Immunologic evaluation of T chronic lymphocyte leukemia cells· Correlations among phenotype, functional activities, and morphology. Blood 59:688, 1982

5 Tagawa S, Taniguchi N, Tokumine Y, Tamaki T, Konishi I, Kanayama Y, Inoue R, Machii T, Kitani T OKM1 positive T cell leukemias· Relationships among morphology, phenotype and functional activities. Cancer 57:1507, 1986

6 Quinti I, Pacilli L, Zoli V, De Sanctis G, Mannella E, Bonomo G, De Laurenzi A, Pandolfi F: Unusual phenotype (Leu 7$^+$, OKT4$^+$, OKM1$^+$) expression by cells from a patient with an abnormal expansion of granular lymphocytes. Acta Haematol 71:310, 1984

7. Reynolds CW, Foon KA: Tγ-lymphoproliferative disease and related disorders in humans and experimental animals: A review of the clinical, cellular, and functional characteristics Blood 64:1146, 1984

8. Kay HD, Bonnard GD, West WH, Herberman RB: A functional comparison of human Fc-receptor-bearing lymphocytes active in natural cytotoxicity and antibody-dependent cellular cytotoxicity. J Immunol 118·2058, 1977

9. Bolhuis RLH, Schuit HRE, Nooyen AM, Ronteltap CPM. Characterization of natural killer (NK) cells and killer (K) cells in human blood: Discrimination between NK and K cell activities. Eur J Immunol 8:731, 1978

10. Van de Griend RJ, Berge IT, Tanke HJ, Roos D, Schellekens PTA, Melief CJM, Zeijlemaker WP, Astaldi A: Characterization of two subsets of Tγ cells J Immunol 128:1979, 1982

11 Timonen T, Ortaldo JR, Herberman RB: Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153·569, 1981

12. Kasahara T, Djeu JY, Dougherty SF, Oppenheim JJ. Capacity of human large granular lymphocytes (LGL) to produce multiple lymphokines. Interleukin 2, interferon, and colony stimulating factor. J Immunol 131.2379, 1983

13. Ortaldo JR, Mason AT, Gerard JP, Henderson LE, Farrar W, Hopkins III RF, Herberman RB, Rabin H: Effects of natural and recombinant IL2 on regulation of IFNγ production and natural killer activity: Lack of involvement of the Tac antigen for these immunoregulatory effects. J Immunol 133.779, 1984

14. Scala G, Allavena P, Djeu JY, Kasahara T, Ortaldo JR, Herberman RB, Oppenheim JJ: Human large granular lymphocytes are potent producers of interleukin-1. Nature 309·56, 1984

15. Mangan KF, Hernett ME, Matis SA, Winkelstein A, Abo T: Natural killer cells suppress human erythroid stem cell proliferation in vitro. Blood 63:260, 1984

16 Phillips JH, Babcock GF: NKP-15· A monoclonal antibody reactive against purified human natural killer cells and granulocytes Immunol Lett 6:143, 1983

17. Suzuki R, Handa K, Itoh K, Kumagai K: Natural killer (NK) cells as a responder to interleukin 2 (IL2). I. Proliferative response and establishment of cloned cells. J Immunol 130:981, 1983

18 Ades EW, Hinson A, Marder P, Butler LD: Potentiation of Leu 11$^+$ natural cytotoxicity by human interleukin 2. Clin Immunol Immunopathol 32·119, 1984

19. Lanier LL, Benike CJ, Phillips JH, Englemen EG· Recombinant interleukin 2 enhanced natural killer cell-mediated cytotoxicity in human lymphocyte subpopulations expressing the Leu 7 and Leu 11 antigens. J Immunol 134.794, 1985

20. Itoh K, Tilden AB, Kumagai K, Balch CM. Leu 11$^+$ lymphocytes with natural killer (NK) activity are precursors of recombinant interleukin 2 (rIL2)-induced activated killer (AK) cells. J Immunol 134:802, 1985

21 Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA: Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 155·1823, 1982

22. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita K, Shirakawa S, Miyoshi I: Adult T-cell leukemia: Antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci USA 78:6476, 1981

23. Miyoshi I, Kubonishi I, Yoshimoto S, Akagi T, Ohtshuki Y, Shiraishi Y, Nagata K, Hinuma Y: Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. Nature 294:770, 1981

24. Tagawa S, Konishi I, Kuratune H, Katagiri S, Taniguchi N, Tamaki T, Inoue R, Kanayama Y, Tsubakio T, Machii T, Yonezawa T, Kitani T. A case of T-cell chronic lymphocytic leukemia (T-CLL) expressing a peculiar phenotype (E$^+$, OKM1$^+$, Leu 1$^+$, OKT3$^-$, and IgGEA$^-$). Cancer 52 1378, 1983

25. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. 1. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. J Immunol 126:1393, 1981

26. Naruo K, Hinuma S, Kato K, Koyama M, Tada H, Shiho O, Tsukamoto K. Comparison of the biological properties of purified natural and recombinant human interleukin-2. Biochem Biophys Res Commun 128:257, 1985

27 Tsujimoto M, Tarutani T, Ogawa K, Akashi K, Okubo Y, Higashi N· Production of human interferon-γ in serum-free medium. Infect Immun 41:181, 1983

28. Kadish AS, Tansey FA, Yu GSM, Doyle AT, Bloom BR· Interferon as a mediator of human lymphocyte suppression. J Exp Med 151·637, 1980

29. Grimm E, Bonavida B: Mechanism of cell-mediated cytotoxicity at the single cell level, I. Estimation of cytotoxic T lymphocyte frequency and relative lytic efficiency. J Immunol 123·2861, 1979

30. Targen S, Dorey F: Interferon activation of "Prespontaneous killer" (PRE-SK) cells and alteration in kinetics of lysis of both "PRE-SK" and active SK cells. J Immunol 124.2157, 1980

31. Payne CM, Glasser L: A quantitative ultrastructural study of peripheral blood lymphocytes containing parallel tubular arrays in Epstein-Barr virus and cytomegalovirus mononucleosis. Am J Pathol 106.71, 1982

32. Kanayama Y, Hiraoka A, Konishi I, Machii T, Tamaki T, Kanakura Y, Yonezawa T, Tarui S, Kitani T: Ultrastructure of normal human T cell subpopulations Parallel tubular arrays in Tγ lymphocytes and clustered dense bodies in Tμ lymphocytes Acta Haematol 70:220, 1983

33 Sundqvist KG, Otteskog P, Wanger L, Thorstensson R, Utter G: Morphology and microfilament organization in human blood

lymphocytes. Effects of substratum and mitogen exposure. Exp Cell Res 130:327, 1980

34. Rambaldi A, Pelicci P, Allavena P, Knowles II DM, Rossini S, Bassan R, Barbui T, Dalla-Favera R, Mantovani A: T cell receptor β chain gene rearrangements in lymphoproliferative disorders of large granular lymphocytes/natural killer cells. J Exp Med 162.2156, 1985

35 Phillips JH, Lanier LL: A model for the differentiation of human natural killer cells. Studies on the in vitro activation of Leu-11$^+$ granular lymphocytes with a natural killer-sensitive tumor cell, K562 J Exp Med 161 1464, 1985

36 Sztein MS, Steeg PS, Johnson HM, Oppenheim JJ. Regulation of human peripheral blood monocyte DR antigen expression in vitro by lymphokines and recombinant interferons. J Clin Invest 73:556, 1984

37. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor. Nature 300:267, 1982

38. Welte K, Andreeff M, Platzer E, Holloway K, Rubin BY, Moore MAS, Mertelsmann R: Interleukin 2 regulates the expression of Tac antigen on peripheral blood T lymphocytes. J Exp Med 160:1390, 1984

39. Trinchieri G, Matsumoto-Kobayashi M, Clark SC, Seehra J, London L, Perussia B: Response of resting human peripheral blood natural killer cells to interleukin 2. J Exp Med 160:1147, 1984

40. Pandolfi F, Capobianchi M, Matricardi P, Facchini J, Bonomo G, DeRossi G, Semenzato G, Fiorilli M, Dianzani F, Aiuti F· Impaired gamma interferon production by cells from patients with lymphoproliferative disorders of mature T and NK cells. Scand J Immunol 21.315, 1985

41 Zoumbus NC, Gascon P, Djeu JY, Young NS· Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo Proc Natl Acad Sci USA 82:188, 1985

42. Hedrick SM, Cohen DI, Nielsen EA, Davis MM: Isolation of cDNA clones encoding T cell-specific membrane-associated proteins Nature 308 149, 1984

43 Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ· Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 313:776, 1985

44. Foa R, Pelicci P, Migone N, Lauria F, Pizzolo G, Flug F, Knowles PM II, Dalla-Fevera R· Analysis of T-cell receptor beta chain (Tβ) gene rearrangements demonstrates the monoclonal nature of T-cell chronic lymphoproliferative disorders. Blood 67.247, 1986

45. Lanier LL, Cwirla S, Federspiel N, Phillips JH: Human natural killer cells isolated from peripheral blood do not rearrange T cell antigen receptor β chain genes J Exp Med 163.209, 1986

Purification and Functional Evaluation of Mature Neutrophils From Human Bone Marrow

By Roger L. Berkow and Robert W. Dodson

Human myeloid maturation proceeds within the bone marrow and results in a mature neutrophil that is released into the peripheral circulation. Previous reports have indicated that neutrophils from bone marrow demonstrate decreased adherence, impaired phagocytosis, and decreased nitroblue tetrazolium dye reduction when stimulated. Due to lack of a suitable method for isolating purified bone marrow neutrophils, these studies have been performed by microscopic techniques. We now report a method for isolating 1 × 10^8 neutrophils [bands plus polymorphonuclear leukocytes (PMNs)] from 10 mL of bone marrow aspirate sample. By means of a discontinuous Percoll-gradient centrifugation through densities of 1.085, 1.095, and 1.10 g/mL a leukocyte-rich suspension of bone marrow can be separated into three leukocyte layers. By combining the lower two leukocyte layers (M2/3), a population of neutrophils consisting of 26% bands and 63% PMNs is seen. When compared with peripheral blood PMNs, these bone marrow neutrophils had a lower alkaline phosphatase activity, decreased ingestion of Oil Red O-coated particles, impaired superoxide release on stimulation with the chemotactic peptide Fmet-leu-phe (FMLP) or the tumor promotor phorbol myristate acetate (PMA), and less activity of the NADPH-dependent oxidase. These results indicate that morphologically mature neutrophilic cells within the bone marrow exist in a still functionally immature state.

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NEUTROPHIL maturation is characterized by the sequential appearance of primary and secondary granules, increased nuclear segmentation, and release of the mature polymorphonuclear leukocytes (PMNs) from the bone marrow (BM) into the peripheral circulation.[1] On interaction with various agents, the circulating PMNs can be activated to adhere to surfaces,[2] move or chemotax,[3] phagocytize opsonized particles,[4] reduce molecular oxygen to toxic species,[5] release granule contents,[5] and aggregate.[6] The development of these functional responses permits the PMNs to protect the host effectively against invading organisms.

Previous reports have indicated that neutrophils from BM demonstrate decreased adherence to surfaces, are poorly deformable, have an impaired ability to phagocytize opsonized particles, and a decreased oxidative burst upon stimulation.[7-9] In addition, neutrophil alkaline phosphatase (AP) activity has been shown to be decreased in myeloid cells isolated from BM as compared with those found in the peripheral blood (PB).[10-13] This membrane-bound enzyme is believed to be an indicator of neutrophil maturity.[14]

Due to lack of a suitable method for isolating large quantities of purified BM neutrophils, many studies investigating the maturation of neutrophil function have been performed by microscopic techniques, using mixed leukocyte suspensions derived from BM aspirate samples or dried smears of the BM sample.[7-9] To circumvent this problem and permit biochemical measurements of PMN functional maturation, studies have been performed on myeloid cells isolated from patients with acute and chronic myelogenous leukemia, or using the HL-60 promyelocytic leukemia cell line, which can be induced to differentiate toward mature granulocytes when treated with dimethyl sulfoxide or retinoic acid.[15-25] These studies have demonstrated that immature neutrophilic forms have deficient phagocytic capacity, lower oxidative burst, impaired bacterial killing, and decreased chemotaxis as compared with equal numbers of mature circulating neutrophils.[23-26] Studies with HL-60 cells have shown that neutrophilic functional responses are deficient in the uninduced cells but, as differentiation proceeds, alterations in surface proteins and receptors occur that are accompanied by increased physiologic responses.[15-22] The abnormal nature of these cells, however, makes it difficult to relate these findings to the maturation of functional events in normal BM myeloid cells. In addition, most of these studies have not adequately evaluated the functional nature of the mature (band plus PMNs) BM neutrophil.

In this article, we describe a method for isolating large numbers of purified mature (band + PMNs) and immature (blasts, myelocytes, and metamyelocytes) neutrophilic cells from BM. The ability to isolate 10^8 mature neutrophils from a 10-mL BM aspirate sample permitted us to use biochemical techniques to demonstrate that morphologically mature neutrophils from the BM have decreased phagocytosis, slower superoxide anion generation on stimulation, decreased NADPH oxidase activity, and less AP activity than do neutrophils isolated from the PB of the same donor.

MATERIALS AND METHODS

Materials used in these studies include Dextran and Percoll (Pharmacia, Piscataway, NJ); acid citrate dextrose formula A (ACD) (Fenwall Labs, Deerfield, Ill); F-met-leu-phe, cytochrome C, superoxide dismutase, *N*-ethyl-maleimide, *p*-nitrophenyl phosphate, guaiacol, Oil Red O, nitroblue tetrazolium dye, L-propanol, Folin and Ciocalteu's phenol reagent, dimethyl sulfoxide, Triton X-100, NADPH (Sigma, St Louis); Phorbol myristate acetate (Consolidated Midland Corp, Brewster, NY); *E coli* lipopolysaccharide (Difco, Detroit); and arachidonic acid (Nuchek Prep Inc, Elysian, Minn).

Gradient preparation. Isoosmotic Percoll solutions with densities of 1.10, 1 095, and 1 085 g/mL were prepared from Percoll

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(density 1.30 g/mL), 10 × phosphate-buffered saline (PBS), and distilled water, according to instructions supplied by Pharmacia.[27] The pH of these solutions was adjusted to 7.4, and then was filter-sterilized. Discontinuous gradients were made by the addition of these solutions to 15-mL polypropylene centrifuge tubes. The bottom layer consisted of 4 mL of the 1.10 g/mL Percoll solution Two additional layers of 3 mL each with a density of 1.095 and 1.085 g/mL were then carefully applied. All solutions were allowed to come to room temperature prior to centrifugation.

Neutrophil preparation. Isolation of mature neutrophils from the PB or BM of healthy volunteers was performed simultaneously. Our research was performed in accordance with the Declaration of Helsinki and the University of Alabama at Birmingham Committee for Human Investigations. After appropriate skin cleansing, 10 mL of BM was aspirated from the posterior iliac crest into a syringe containing 1.7 mL of ACD. Slides were prepared for differential counts and the remaining BM was diluted to 20 mL with 0.9% NaCl This cell suspension was forced through needles with decreasing internal diameter (19, 20, and 21 gauge) to disrupt any remaining fragments. Five milliliters of 6% Dextran (70) in 0.9% NaCl was added to allow erythrocyte sedimentation. After 60 minutes at 25 °C the leukocyte-rich supernatant (LRS) was removed and centrifuged at 400 g at 4 °C; the supernatant was discarded, and the pellet was suspended with 8 to 10 mL of 0.9% NaCl.

For the isolation of neutrophils from PB, the following modifications were made. Whole blood (45 mL) was obtained by venipuncture, anticoagulated with 14.5% ACD, and mixed with 10 mL of Dextran as described. The leukocyte-rich supernatant (LRS) was removed after 1 hour at 25 °C and centrifuged as described. The pellet was suspended in 0.9% NaCl to form a leukocyte suspension.

Four to 5 mL of the leukocyte suspension, containing $\sim 5 \times 10^7$ nucleated cells/mL, obtained from BM or PB was layered over the temperature-equilibrated discontinuous Percoll gradient and centrifuged at 700 g at 25 °C for 30 minutes in a swinging bucket rotor in a Beckman TJ-6R centrifuge The rotor speed was allowed to develop slowly, and no brake was used at the completion of the centrifugation. Three leukocyte bands appeared. These were removed with a polypropylene transfer pipette and washed in 0.9% NaCl. Due to the presence of small amounts of erythrocyte contamination in the BM leukocyte bands, all bands were exposed for 20 seconds at 25 °C to distilled water for lysis of erythrocytes, with reconstitution of osmolarity with 3.5% NaCl Peripheral blood neutrophils were similarly treated. The cells were then washed twice in PBS with 1 mmol/L of $CaCl_2$, 1 mmol/L of $MgSO_4$, and 1 mg/mL glucose (KRPG) and resuspended for study.

Cell number and morphology Cell number and volume were determined as previously described[30] using a Coulter model ZF cell counter connected to a Coulter 1000 channelyzer equipped with an XY recorder Morphology was determined from Wright's stained cytocentrifuge preparations All differential counts were of 200 cells performed in duplicate.

Superoxide release. Neutrophil superoxide anion (O_2^-) release on stimulation with the chemotactic peptide, F-met-leu-phe (FMLP); the tumor promotor, phorbol 12-myristate 13-acetate (PMA); or serum-treated zymosan was determined by the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome C by the method of Babior and co-workers[28] to determine cumulative O_2^- release, or by the method of Cohen and Chovaniec[29] to assess the kinetics of O_2^- production. O_2^- release was calculated using our previously described extinction coefficient.[30]

Activity of the NADPH-dependent oxidase was determined by the SOD-inhibitable reduction of ferricytochrome C in a cell-free neutrophil sonicate incubated with 82 µmol/L of arachidonic acid in the presence of NADPH, according to the methods of Curnutte[32] and McPhail and colleagues.[33]

Neutrophil enzyme content The cell content of the neutrophil membrane constituent AP was determined by the method of DeChatelet and Cooper using *p*-nitrophenyl-phosphate as the substrate.[31] The assay time was 30 minutes. The results obtained are expressed as micromoles of *p*-nitrophenyl phosphate hydrolyzed for each 30 minutes per milligram of protein. Protein content was determined by the method of Lowry and colleagues.[34]

The primary granule constituent myeloperoxidase (MPO) was determined by our previously defined method using the oxidation of guaiacol by myeloperoxidase in the presence of hydrogen peroxide.[35] The change in absorbance was followed at 470 nm at 25 °C. Results are expressed as micromoles of guaiacol oxidized per minute per milligram of protein.

Neutrophil phagocytosis Neutrophil phagocytosis was determined by the ingestion of Oil Red O-coated lipopolysaccaride particles opsonized with pooled human serum according to the method of Stossel [36] The reaction time was 5 minutes at 37 °C, results are expressed as milligrams of ORO per 1 × 10^7 PMNs/5 min All assays were performed in triplicate.

Statistical analysis was made by Student's *t* test of paired data, using a two-tailed evaluation

RESULTS

Isolation of bone marrow neutrophils. Figure 1 reveals the appearance of the Percoll gradient before and after centrifugation of the LRS obtained from BM aspirates. Four cell bands appeared (Table 1). M1, at the interface of the saline and 1.085-g/mL layer, contained 37% myelocytes, promyelocytes, and blasts, and 30% metamyelocytes with only 15% mature neutrophils (band form plus segmented PMNs). M2, at the interface of the 1.085-g/mL and 1.095-g/mL layers covered ~5 to 8 mm of the gradient. It contained 90% mature neutrophils and accounted for 69.9% of the total mature neutrophils isolated from the combined M2/M3 fraction. Also seen in the M2 layer were 6% metamyelocytes, 2% myelocytes, and 2% mature eosinophils. M3, at the interface of the 1.095- and 1.10-g/mL layers covered ~5 mm, contained 91% mature neutrophils, and accounted for 30.1% of the mature neutrophils in the combined M2/M3 fraction. Also contained in the M3 layer were 1% myelocytes, 2% metamyelocytes, 5% eosinophils, and 1%

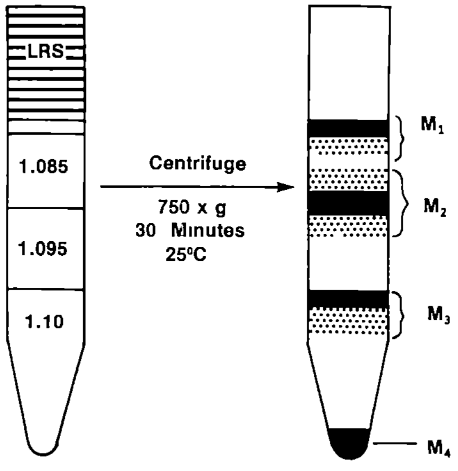


Fig 1. Diagram of Percoll gradient before centrifugation and the distribution of bone marrow leukocyte layers after centrifugation.

Table 1. Percentage of Cell Types in Bone Marrow Fractions

F	My	MM	B	P	Eos	Ly	O	Yield P + B (% from LRS)
M_1	37	30	11	4	ND	14	4	14.6
M_2	2	6	33	57	2	ND	ND	—
M_3	1	2	12	79	5	ND	1	—
$M_{2/3}$	2	5	26	63	4	ND	ND	68.9
LRS	14	16	16	31	2	6	15	100.0

F, bone marrow fraction, My, myelocyte; MM, metamyelocyte, B, band form, P, polymorphonuclear leukocytes; Eos, eosinophil (mature); Ly, lymphocyte, O, other (erythrocyte precursors, myeloblasts); ND, none detected, LRS, leukocyte-rich supernatant. Results are the mean of nine separate separations.

nucleated erythroid precursors. M4 was represented as the RBC pellet that contained variable numbers of leukocytes. To obtain the greatest yield of mature neutrophils, the M2 and M3 layers were combined. This combined layer contained 89% mature neutrophils, 4% eosinophils, 5% metamyelocytes, and 2% myelocytes and yielded a mean of 1.2 ± 0.2 × 10^8 mature neutrophils/10 mL of BM aspirate sample. The M2/M3 fraction was used in all further studies and is referred to as BM neutrophils. These were noted to be 99% viable by exclusion of 0.2% (wt/vol) trypan blue and represented 68.9% of the mature neutrophils present in the LRS applied to the gradient.

When the LRS of PB was subjected to the same discontinuous Percoll-density centrifugation, three distinct leukocyte bands were observed that corresponded in position to the BM bands already described. Lymphocytes and monocytes were confined to the PB_1 layer. PB_2 contained 95% PMNs and 5% band forms and was most frequently of low yield. The PB_3 layer was noted to contain 95% PMNs, 4% eosinophils, and 1% band forms. The PB_4 layer containing the RBC pellet was noted to have variable numbers of eosinophils with minimal contamination by PMNs. As with the BM, the PB_2 and PB_3 layers were combined and yielded 1 to 2 × 10^8 mature neutrophils/100 mL of whole blood initially obtained. Viability was always >99%.

Neutrophil volume. We previously reported that PB PMNs can be separated into at least six fractions based on differences in mean cell volume.[37] To determine if volume variation in PB PMNs could be related to PMN age we assessed cell volume, as described in the Materials and Methods section, in the BM and PB neutrophils. Although the M2 fraction appeared to be slightly larger than PB neutrophils, when the M2/M3 fraction was compared with PB neutrophils, no consistent difference in volume was detectable (data not shown).

Neutrophil enzyme content. Based on histochemical staining, it has long been established that the AP content of BM neutrophilic cells is less than that of PB cells.[10-13] This has also been demonstrated by the observation that biochemically measured neutrophil AP decreases in the PB after administration of corticosteroids to release the BM neutrophil pool.[38] Figure 2 shows that Percoll-purified BM PMNs isolated as described in the Materials and Methods section have 45% ± 4.4% of the biochemically measured AP activity as is noted in the PB neutrophils ($P < .001$).

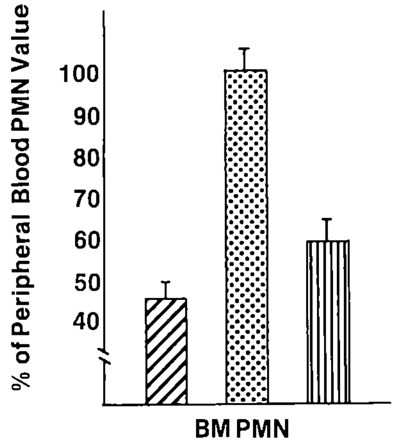


Fig 2. ▨, Alkaline phosphatase activity; ░, myeloperoxidase activity; and ▥, oil red O phagocytosis rate of bone marrow neutrophils isolated as described in the Materials and Methods section. Results are expressed as a percentage of the peripheral blood value: 100% values represent 1.07 ± .11 μmol/30 min/mg of protein, .657 ± .085 μmol/min/mg of protein, and .397 ± .082 mg ORO/10^7 PMNs/5 min for alkaline phosphatase, myeloperoxidase, and phagocytic rate, respectively. Mean ± SEM, n = 7 to 10.

Koeffler and co-workers, using the HL-60 promyelocytic leukemic cell line, showed that synthesis of the primary granule constituent myeloperoxidase decreased as the cell matured.[15,39] This may imply that BM neutrophils, being less mature than circulating neutrophils, should have greater MPO activity than PB neutrophils. Figure 2 reveals no difference between these populations in MPO activity, which indicates that MPO synthesis is complete by the time the neutrophilic cells reach the band form stage of development, and degradation of activity does not accompany further maturation.

Phagocytosis by bone marrow neutrophils. Altman and Stossel reported that neutrophilic cells within the BM have a decreased capacity to ingest particles compared to neutrophils from PB.[9] These data are supported by the observation of decreased phagocytosis by leukemic myeloid cells.[24,25] Figure 2 shows that when ORO-coated lipopolysaccharide particles that had been opsonized with human serum were used, BM neutrophils ingested 59.5% ± 4.6% of the milligrams of Oil Red O as did PMNs from PB ($P < .01$).

Release of superoxide anion. By examining whole BM samples, Zakhireh and Root showed that immature neutrophils from BM had decreased reduction of nitroblue tetrazolium dye, indicating a decreased oxidative capacity.[8] No study has directly evaluated the superoxide release of mature neutrophils isolated from BM.

Table 2 shows that BM neutrophils released 23.3%, 34.9%, and 24.0% of the O_2^- released by similar numbers of PB neutrophils, in a 5-minute assay, when 10^{-6}, 10^{-7}, and 10^{-8} mol/L of FMLP, respectively, was the stimulant. To determine if the decrease in O_2^- release observed was due to a delay in initiation of the respiratory burst or due to an altered maximal velocity and duration of O_2^- release we assessed the kinetics of O_2^- release. Figure 3 shows that in a representative experiment the lag time to initiation of the respiratory burst was similar for both BM and PB neutrophils. BM

Table 2. Release of O_2- by PB and BM Neutrophils With FMLP as Stimulant

FMLP	PB	BM
1×10^{-6}mmol/L	21 9 ± 2 8	5 1 ± 1.0*
1×10^{-7}mmol/L	18.3 ± 246	6.4 ± 3.4*
1×10^{-8}mmol/L	7 3 ± 3 0	1.8 ± 0.6

FMLP, F-met-leu-phe; PB, peripheral blood; BM, bone marrow.

Nanomoles of O_2-/2 5 × 10^6 neutrophils/5 minutes; M ± SEM; n = 3 to 9.

*$P < .05$ *v* PB, paired *t* test.

neutrophils, however, demonstrated a slower maximal rate of O_2^- production regardless of the concentration of FMLP used as the stimulant. Also apparent from Fig 3 is that the duration of O_2^- release (time to reach plateau) was the same for both BM and PB neutrophils. These data indicate that the slower O_2^- release noted in BM neutrophils stimulated with FMLP was not overcome by increasing the agonist concentrations and that the initiation and termination of the respiratory burst was the same for BM and PB neutrophils.

To determine if the impaired O_2^- release by BM neutrophils was unique to the agonist used or due to an intrinsic cellular defect, we assessed the respiratory burst induced by the tumor promotor, PMA, an agonist that activates neutrophils by a different biochemical mechanism than that of FMLP.[40-43] Figure 4 shows a representative experiment which revealed that, as with FMLP, BM neutrophils stimulated with PMA have a slower rate of O_2^- release (48% to 60% of PB neutrophils), regardless of the concentration of stimulant used. The maximal rate of O_2^- release of BM and PB neutrophils is noted in the inset to Fig 4. Although the duration of O_2^- release of BM neutrophils stimulated with PMA was slightly longer than for PB neutrophils (as shown in Fig 4), allowing the reaction to proceed for times up to 30 minutes did not alter our findings. Lag time to initiation of O_2^- release was not significantly different between BM and PB neutrophils, 58.5 ± 7.9, 84.25 ± 18.0 and 194 ± 12.5 seconds *v* 58.5 ± 7.9, 76.5 ± 19.5 and 218 ± 18.2 s at 5, 2.5 and 1 × 10^{-8} mol/L of PMA, respectively, for BM and PB neutrophils (M ± SEM, n = 5). In addition, we also observed a similar slowing of O_2^- release when serum-treated zymosan particles were used as the stimulus (data not shown).

Because activation of the NADPH-dependent oxidase represents the final pathway for the reduction of molecular oxygen to O_2^- by all neutrophil agonists,[32,40,41] we measured the activity of this enzyme system in cell-free neutrophil sonicates in the presence of arachidonic acid and NADPH. Figure 5 shows that BM neutrophils contained only 47% of the NADPH-dependent oxidase activity as did PB neutrophils (4.40 ± 0.4 *v* 9.36 ± 1.20 nmol O_2^-/min/mg of cell protein for BM and PB neutrophils, respectively, M ± SEM, n = 9).

Based on these results, it is likely that a deficiency of NADPH oxidase activity in BM neutrophils is the cause of the slower rate of O_2^- release noted. To determine if the 26% band forms in the BM neutrophil suspension or a subpopulation of BM-segmented PMNs were not effectively producing O_2^- on stimulation, we assessed the reduction of nitroblue tetrazolium dye on addition of PMA (2.5 and 5.0 × 10^{-8} mol/L) by BM neutrophils that were adherent to glass slides. We observed that in the adherent cells, band forms still represented 25% to 30% of neutrophilic cells. Although BM neutrophils were noted to have fewer blue-black granules per cell than did PB neutrophils, 96% of BM neutrophils demonstrated reduction of the dye (data not shown).

DISCUSSION

In a previous publication, we reported that PB PMNs could be separated, by counterflow centrifugal elutriation, into at least six fractions that had increasingly larger mean peak volumes. We demonstrated that the larger PMN fractions had a greater oxidative burst on stimulation with either FMLP or PMA. In addition, larger PMNs were shown to have a greater content of the primary granule constituent myeloperoxidase than did smaller PMNs.[37] Further studies have shown that larger PMNs have a greater phagocytic capacity but lower alkaline phosphatase activity per milligram of protein than that of smaller PMNs.[44] To determine if volume-dependent PMN fractions were related to the age of the PMN or to the time of release from the BM PMN

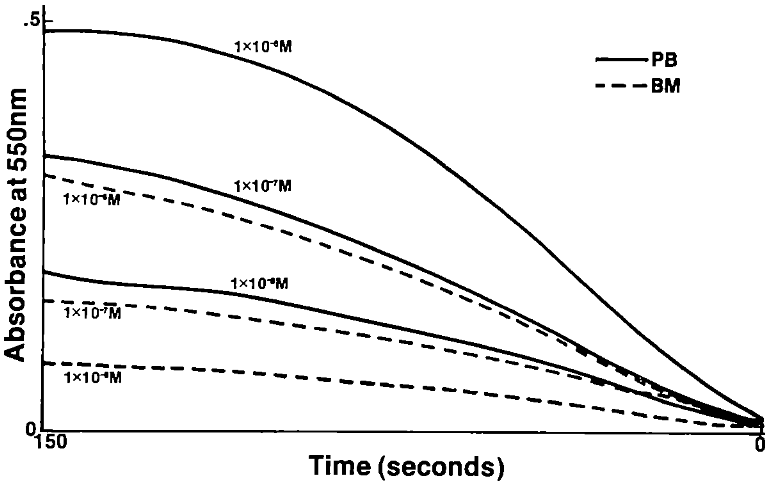
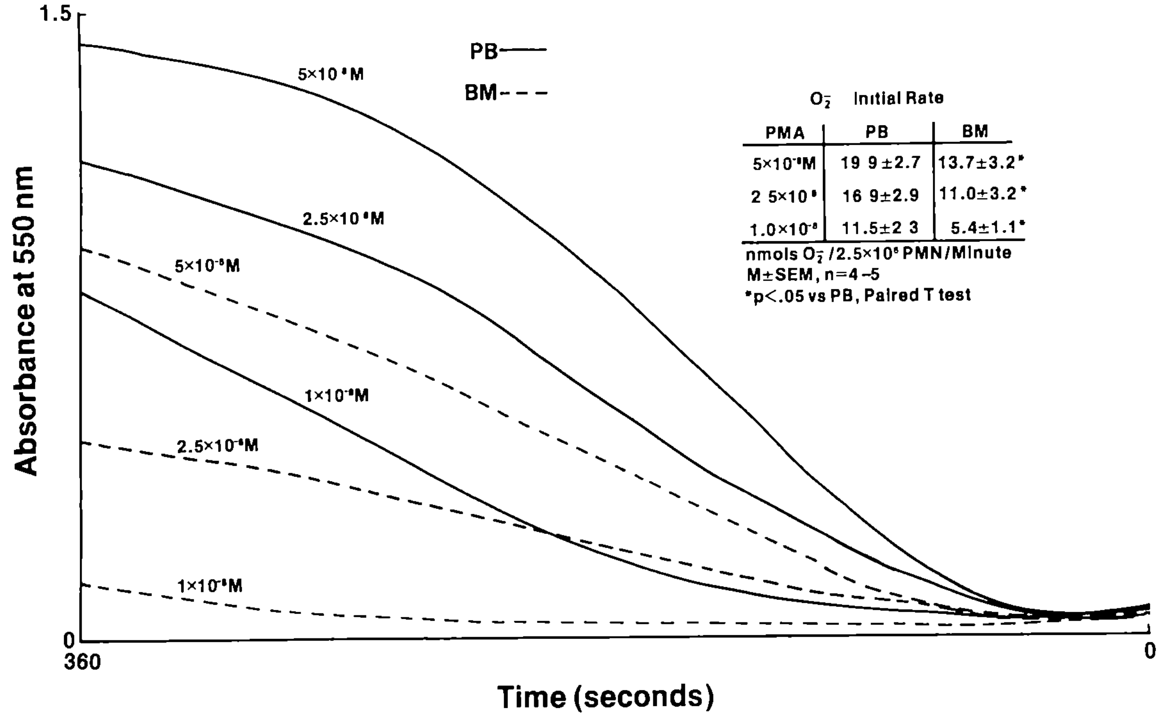


Fig 3. SOD-inhibitable reduction of ferricytochrome C of 2.5 × 10^6 bone marrow (BM) or peripheral blood (PB) neutrophils incubated with the stated concentration of F-met-leu-phe (FMLP), and measured in a double-beam spectrophotometer as the change in absorbance at 550 nm at 37 °C. FMLP is added at time 0. Lag time and maximal rate are determined as described by Cohen and Chovaniec[29].



O_2^- Initial Rate

PMA	PB	BM
5×10^{-8}M	19.9±2.7	13.7±3.2*
2.5×10^{-8}	16.9±2.9	11.0±3.2*
1.0×10^{-8}	11.5±2.3	5.4±1.1*

nmols O_2^-/2.5×10^6 PMN/Minute
M±SEM, n=4–5
*$p<.05$ vs PB, Paired T test

Fig 4. SOD-inhibitable reduction of ferricytochrome C of 2.5 × 10^6 bone marrow (BM) or peripheral blood (PB) neutrophils incubated with the stated concentration of phorbol 12-myristate 13-acetate (PMA), and measured as the change in absorbance at 550 nm at 37 °C in a double-beam spectrophotometer. PMA is added at time 0. The inset shows the maximal rate of O_2^- release expressed as nanomoles of O_2^-/2.5 × 10^6 neutrophils per minute.

storage pool, it was necessary to develop a technique for isolating large quantities of mature BM neutrophils.

Although many studies have examined the isolation of various colony-forming cells from BM,[45–50] few have attempted to purify mature neutrophils from this storage pool. Early attempts to concentrate mature BM neutrophils have been reported by Evans and co-workers.[51] In their study, 8 × 10^7 metamyelocytes, bands, and PMNs with 85% purity were recovered from a 15-mL BM sample by means of sequential centrifugation in 15.6% and then 20% Ficoll. Because no differential counts were reported, the number of bands and PMNs in this preparation cannot be determined. Sato and colleagues[12,52] used discontinuous gradients of Ficoll and sodium metrizoate to isolate postmitotic neutrophils with >70% purity. No further breakdown of this neutrophilic population was given. Using continuous Percoll gradients, Olofsson and colleagues[53] determined the densities of the various neutrophilic cells from human BM and showed that metamyelocytes and mature neutrophils had densities of 1.0799 and 1.0864 g/mL, respectively. By adapting the data reported in these studies, we developed the method previously described for isolating purified BM neutrophils.

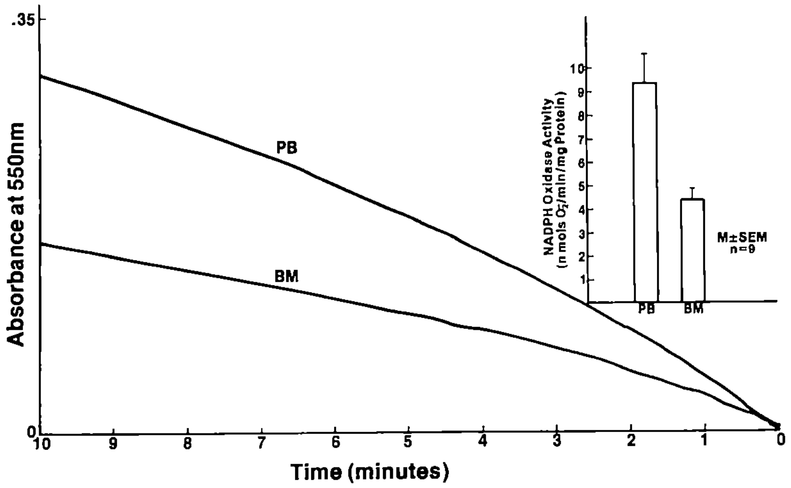


Fig 5. NADPH oxidase activity of bone marrow (BM) or peripheral blood (PB) neutrophil sonicates incubated with NADPH, 82 µmol/L of arachidonic acid, and ferricytochrome C according to the method of Curnutte.[32] Results are given as the reduction of ferricytochrome C followed at 550 nm at 25 °C in a double-beam spectrophotometer. Arachidonic acid is added at time 0 to initiate the reaction. Inset: NADPH oxidase activity of BM or PB neutrophils expressed as nanomoles of O_2^- per milligram of protein per minute M ± SEM, n = 9.

The results presented here indicate that morphologically mature neutrophils, ie, bands plus PMNs, can be isolated in sufficient purity and quantity from small volumes of BM to allow biochemical studies. Several points concerning the separation must be noted. First, in the preliminary work, we found that the density of the uppermost layer was crucial to preventing the contamination of the M2 layer with myelocytes and metamyelocytes. A decrease in density of this layer by only .003 to .005 g/mL led to a considerable contamination of M2. Second, as shown in Table 1, we found that the M2 layer had a higher percentage of band forms as compared with the M3 layer, suggesting that cell density increases as the band form neutrophils mature to PMNs. Third, the layering within the Percoll gradient of the PB neutrophils isolated at the same time as BM neutrophils revealed that most PMNs recovered from the PB_3 layer (data not shown) and thus were demonstrating a somewhat higher mean density than were the mature neutrophils isolated from the BM. This indicates a continued progression to higher density as the PMN matures in the peripheral circulation. Fourth, the presence of variable RBC contamination in the M2 and M3 layer required the use of a hypotonic lysis procedure to ensure the removal of these elements. Last, despite repeated manipulations of the gradients, we were unable to separate the band forms from the PMNs within layer M2.

As stated previously, earlier studies using microscopic techniques revealed decreased AP activity, ingestion of particles, and reduction of molecular oxygen in BM neutrophils.[7–10] Biochemical studies using the HL-60 cell line demonstrated similar findings.[15–22] The results of the present study reveal that morphologically mature BM neutrophils have only 45% of the biochemically measured AP activity, 60% of the phagocytic capacity, and a slower O_2^- release as compared with PB neutrophils, confirming previous studies using cytochemical techniques.[7–10] These results also confirm the validity of using these parameters as indicators of neutrophil maturity and age. In addition, that the present findings do not correlate with those noted for volume-dependent PMN fractions indicates that volume heterogeneity among PMNs is probably not a reflection of PMN age as previously suspected.[37]

The development of the ability to release superoxide anion is required if the mature neutrophil is to kill bacteria effectively. The formation of this reduction product of molecular oxygen depends, in the PMN, on the activation of an NADPH-dependent oxidase. This oxidase has been shown to be activated by differing mechanisms dependent on the stimulus used.[40,41] As shown in the Results section, BM neutrophils demonstrated a slower rate of O_2^- release with a similar lag time and duration of O_2^- production as compared with PB neutrophils. This slower rate was not overcome by higher concentrations of stimulants, indicating that the results are not due to an altered threshold for stimulation. In addition, the slower rate of O_2^- release of BM neutrophils occurred regardless of the stimulant used. These results suggest that the existence of a subpopulation of nonresponding cells or an alteration of the O_2^- producing mechanism of these cells in some way. The results obtained for nitroblue tetrazolium dye reduction indicate that our findings are not due to a lack of oxygen-reductive capability by the BM bands or a subpopulation of the BM-segmented forms, although the intensity of nitroblue tetrazolium reduction was somewhat less in all BM neutrophils than in PB neutrophils. The data clearly indicate, however, that BM neutrophils contain only 47% of the NADPH oxidase activity contained in PB neutrophils and imply that this deficiency is responsible for the decrease in O_2^- release noted in the BM neutrophils.

Although release of mature neutrophils from BM undoubtedly occurs during the stress of severe bacterial infection, it is questionable if the deficits in function seen in the present study would lead to a physiologic deficiency of host defense. First, it is noted that females who are heterozygous for the gene coding for chronic granulomatous disease do not have significant bacterial infections despite having, on the average, only 50% of the superoxide-generating capacity of normal persons.[54] Second, during bacterial infections, the neutrophils are exposed to various endogenous and bacterial products that may actually augment their responses to subsequent exposures to stimulants; thus, BM neutrophils may have their responses augmented or "primed" on being released during times of infection.[55–57] This is supported by the observation of an increased PMN oxidative burst in patients with active bacterial infections.[58]

In conclusion, we report an effective method for the isolation of large numbers of morphologically mature (bands and segmented) neutrophils from small quantities of BM aspirate samples. We confirmed earlier reports demonstrating decreased AP activity, phagocytosis, and oxidative burst of BM neutrophils. We extended these studies to demonstrate that BM neutrophil O_2^- release was slower, but with a similar lag time and duration of release, as compared with PB neutrophils, regardless of whether FMLP, PMA or serum-treated zymosan was used as the stimulant. Moreover, we demonstrated that this was not due to the increased percentage of neutrophilic band forms or a subpopulation of cells with an inability to produce O_2^- in the BM neutrophils. Finally, we are first to demonstrate a decreased NADPH oxidase activity in morphologically mature BM neutrophils as the cause of the deficient stimulated oxidative burst.

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REFERENCES

1. Bainton DF, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow J Exp Med 134 907, 1971

2 Fehr J, Dahinden C: Modulating influence of chemotactic factor-induced cell adhesiveness on granulocyte function. J Clin Invest 64.8, 1979

3. Snyderman R, Goetzl EJ· Molecular and cellular mechanisms of leukocyte chemotaxis. Science 213.830, 1981

4. Stossel TP: Phagocytosis. N Engl J Med 290:717, 1974

5. Weissmann G, Korchak HM, Perez HD, Smolen JE, Goldstein IM, Hoffstein ST· The secretory code of the neutrophil. J Reticuloendothel Soc 22:687, 1979

6. Oseas RS, Boxer LA, Butterick C, Baehner RL· Differences in polymorphonuclear leukocyte aggregating responses among several species in response to chemotactic stimulation. J Lab Clin Med 96·213, 1980

7 Lichtman MA, Weed RI. Alteration of the cell periphery during granulocyte maturation: Relationship to cell function. Blood 39:301, 1972

8. Zakhireh B, Root RK· Development of oxidase activity by human bone marrow granulocytes. Blood 54:429, 1979

9. Altman AJ, Stossel TP: Functional immaturity of bone marrow bands and polymorphonuclear leucocytes. Br J Haematol 27:241, 1974

10. Trubowitz S, Feldman D, Benante C, Hunt VM: The alkaline phosphatase content of the human polymorphonuclear leukocyte in blood and marrow. Am J Clin Pathol 31(6):481, 1959

11. Trubowitz S, Moschides E, Feldman D: Alkaline phosphatase activity of the polymorphonuclear leukocyte in rapidly induced leukopenia and leukocytosis. J Lab Clin Med 57:747, 1961

12. Sato N, Asano S, Urabe A, Ohsawa N, Takaku F: Induction of alkaline phosphatase in neutrophilic granulocytes, a marker of cell maturity, from bone marrow of normal individuals by retinoic acid. Biochem Biophy Res Commun 131(3)·1181, 1985

13. Wachstein M: Alkaline phosphatase activity in normal and abnormal human blood and bone marrow cells. J Lab Clin Med 31(1).1, 1946

14. Pedersen B: Functional and biochemical phenotype in relation to cellular age of differentiated neutrophils in chronic myeloid leukemia. Br J Haematol 51.339, 1982

15 Koeffler HP. Induction of differentiation of human acute myelogenous leukemia cells: Therapeutic implications. Blood 62.709, 1983

16 Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. J Exp Med 149 969, 1979

17. Kitagawa S, Ohta M, Nojiri H, Kakinuma K, Saito M, Takaku F, Miura Y. Functional maturation of membrane potential changes and superoxide-producing capacity during differentiation of human granulocytes. J Clin Invest 73:1062, 1984

18. Newburger PE, Chovaniec ME, Greenberger JS, Cohen HJ: Functional changes in human leukemia cell line HL-60. J Cell Biol 82:315, 1979

19. Cotter TG, Henson PM: A cell surface differentiation antigen involved in human neutrophil chemotaxis and degranulation Clin Exp Immunol 53.249, 1983

20 Stendahl O, Dahlgren C, Hed J· Physicochemical and functional changes in human leukemic cell line HL-60. J Cell Physiol 112.227, 1982

21. Fontana JA, Wright DG, Schiffmann E, Corcoran BA, Deisseroth AB: Development of chemotactic responsiveness in myeloid precursor cells: Studies with a human leukemia cell line. Proc Natl Acad Sci USA 77(6):3664, 1980

22. Wood PA, Douglas SD: Development of chemotaxis and formyl peptide binding in human promyelocytic leukemia cell line (HL60) (41369). Proc Soc Exp Biol Med 169:421, 1982

23. Glasser L: Leukemic cells as probes for sequential functional differentiation of the human granulocyte. Am J Clin Pathol 79(1).45, 1983

24. Glasser L: Phagocytosis in acute leukemia Cancer 45(6):1365, 1980

25. Olofsson T, Odeberg H, Olsson I. Granulocyte function in chronic granulocytic leukemia II. Bactericidal capacity, phagocytic rate, oxygen consumption, and granule protein composition in isolated granulocytes. Blood 48 581, 1976

26. Rosner F, Valmont I, Kozinn PJ, Caroline L: Leukocyte function in patients with leukemia. Cancer 25(4):835, 1970

27. Anonymous: Percoll—Methodology and Applications. Pharmacia, Inc Piscataway, NJ

28. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanisms The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52:741, 1973

29. Cohen HJ, Chovaniec, ME: Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for continuous assay for monitoring superoxide production and for the study of the activation of the generating system. J Clin Invest 61.1081, 1978

30. Berkow RL, Tzeng DY, Williams LV, Baehner RL: The comparative responses of human polymorphonuclear counterflow centrifugal elutriation and Ficoll-hypaque density centrifugation. I. Resting volume, stimulus-induced superoxide production, and primary and specific granule release J Lab Clin Med 102(5).732, 1983

31 DeChatelet LR, Cooper MR: A modified procedure for the determination of leukocyte alkaline phosphatase Biochem Med 4:61, 1970

32. Curnutte JT. Activation of human neutrophil nicotinamide adenine dinucleotide phosphate, reduced (triphosphopyridine nucleotide, reduced) oxidase by arachidonic acid in a cell-free system. J Clin Invest 75:1740, 1985

33. McPhail LC, Shirley PS, Clayton CC, Snyderman R· Activation of the respiratory burst enzyme from human neutrophils in a cell-free system J Clin Invest 75:1735, 1985

34 Lowry OH, Rosebrough WJ, Farr AL, Randall KJ· Protein measurement with the Folin-phenol reagent J Biol Chem 193:265, 1951

35. Paul BB, Strauss RR, Jacobs AA, Sbarra AJ: Function of H_2O_2, myeloperoxidase, and hexose monophosphate shunt enzymes in phagocytizing cells from different species. Infect Immun 1:338, 1970

36 Stossel TP: Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. Blood 42:121, 1973

37. Berkow RL, Baehner RL. Volume-dependent human blood polymorphonuclear leukocyte heterogeneity demonstrated with counterflow centrifugal elutriation. Blood 65.71, 1985

38. Fehr J, Grossmann HC· Disparity between circulating and marginated neutrophils Evidence from studies on the granulocyte alkaline phosphatase, a marker of cell maturity. Am J Hematol 7·369, 1979

39 Koeffler HP, Ranyard J, Pertcheck M· Myeloperoxidase. Its structure and control of its gene expression during myeloid differentiation Blood 62.83a, 1983 (suppl 1)

40. McPhail LC, Snyderman R: Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli Evidence that the same oxidase is activated by different transductional mechanisms J Clin Invest 72:192, 1983

41. McPhail LC, Henson PM, Johnston RB: Respiratory burst enzyme in human neutrophils: Evidence for multiple mechanisms of activation. J Clin Invest 67:710, 1981

42 Wolfson M, McPhail LC, Nasrallah VN, Snyderman R: Phorbol myristate acetate mediates redistribution of protein kinase C in human neutrophils: Potential role in the activation of the respiratory burst enzyme J Immunol 135(3):2057, 1985

43 Volpi M, Naccache PH, Molski TFP, Shefcyk J, Huang CK, Marsh ML, Munoz J, Becker EL, Sha'afi RI: Pertussis toxin inhibits fMet-leu-phe- but not phorbol ester-stimulated changes in rabbit neutrophils· Role of G proteins in excitation response coupling. Proc Natl Acad Sci USA 82:2708, 1985

44 Berkow RL: Volume dependent polymorphonuclear leuko-

cyte fractions exist but do not represent neutrophils of varied ages J Cell Biol 101:240a, 1985

45. Wells JR, Opelz G, Cline MJ: Characterization of functionally distinct lymphoid and myeloid cells from human blood and bone marrow. I. Separation by a buoyant density gradient technique. J Immunol Methods 18:63, 1977

46. Wells JR, Opelz G, Cline MJ: Characterization of functionally distinct lymphoid and myeloid cells from human blood and bone marrow. II. Separation by velocity sedimentation. J Immunol Methods 18:79, 1977

47. Frickhofen N, Heit W, Heimpel H: Enrichment of hematopoietic progenitor cells from human bone marrow on Percoll density gradients Blut 44:101, 1982

48. Inoue T, Carsten AL, Cronkite EP, Kelley JET: Separation and concentration of murine hematopoietic stem cells (CFUs) using a combination of density gradient sedimentation and counterflow centrifugal elutriation Exp Hematol 9(6):563, 1981

49. Meyskens FL, Kiefer CA, Holmes DK, Gerner EW Separation of normal human bone marrow cells by counterflow centrifugal elutriation. I. Morphological analysis and subfractionation of neutrophilic granulocytes. Exp Hematol 7(8):401, 1979

50. Jemionek JF, MacVittie TJ, Byrne PJ, Schein PS, Walden DA: Fractionation of mammalian bone marrow by counterflow centrifugation elutriation using a continuous albumin gradient: Analysis of granulocyte-macrophage colony-forming units Br J Haematol 50:257, 1982

51 Evans WH, Wolf MM, Chabner BA: Concentration of immature and mature granulocytes from normal human bone marrow. Proc Soc Exp Biol Med 146:526, 1974

52 Sato N, Mori M, Oshimura M, Ueyama Y, Miwa T, Ohsawa N, Kosaka K, Asano S: Factor(s) responsible for the increase in alkaline phosphatase activity of postmitotic granulocytes from normal individuals and patients with chronic myeloid leukemia Blood 59:141, 1982

53. Olofsson T, Gartner I, Olsson I: Separation of human bone marrow cells in density gradients of polyvinylpyrrolidone coated silica gel (Percoll). Scand J Haematol 24 254, 1980

54. Tauber AI, Borregaard N, Simons E, Wright J: Chronic granulomatous disease: A syndrome of phagocyte oxidase deficiencies Medicine 62 286, 1983

55. McPhail LC, Clayton CC, Snyderman R: The NADPH oxidase of human polymorphonuclear leukocytes J Biol Chem 259(9) 5768, 1984

56. Gay JC, Beckman JK, Brash AR, Oates JA, Lukens JN: Enhancement of chemotactic factor-stimulated neutrophil oxidative metabolism by leukotriene B_4 Blood 64:780, 1984

57. Dahinden C, Galanos C, Fehr J: Granulocyte activation by endotoxin. I. Correlation between adherence and other granulocyte functions, and role of endotoxin structure on biologic activity J Immunol 130:857, 1983

58. Barbour AG, Allred CD, Solberg CO, Hill HR: Chemiluminescence by polymorphonuclear leukocytes from patients with active bacterial infection. J Infect Dis 141:14, 1980

Characterization of Host Cells Involved in Resistance to Marrow Grafts in Dogs Transplanted From Unrelated DLA-Nonidentical Donors

By Robert F. Raff, H. Joachim Deeg, Thomas P. Loughran, Jr, Theodore C. Graham,
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A canine model of marrow transplantation was used to further define the host cells mediating resistance to marrow engraftment. Recipient dogs were given 9.2 Gy of total body irradiation followed by marrow infusion from unrelated DLA-nonidentical donors. No postgrafting immunosuppression was given. At three and ten days posttransplantation recipient marrow and peripheral blood cells were obtained and characterized by the following in vitro studies: (a) morphologic analysis; (b) phenotypic analysis with monoclonal antibodies; (c) assays for natural killer cell (NK) activity; and (d) cocultures with donor marrow to study the effect on donor CFU-GM growth. Daily differential cell counts revealed a proliferation of peripheral blood mononuclear cells approximately eight days posttransplant. By day 10 surviving host cells were uniformly large granular lymphocytes which were phenotypically of T cell lineage, had NK activity, and were capable of suppressing donor marrow CFU-GM growth. Mononuclear cells from dogs given total body irradiation only and no marrow infusion (radiation control group), did not suppress CFU-GM growth when cocultured with marrow from unrelated DLA-mismatched dogs. These results suggest that radioresistant host cells with the morphology of large granular lymphocytes and NK activity and which proliferate in response to the infused donor marrow cells mediate resistance to DLA-nonidentical marrow grafts. It remains to be determined, however, whether in vitro functional studies reflect the mechanisms involved in vivo.

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PREVIOUS STUDIES in a canine model of marrow transplantation have investigated factors which influence resistance to engraftment and marrow graft rejection. These studies have established the importance of DLA-encoded histocompatibility antigens, or antigens in linkage with but different from known DLA antigens, for engraftment in related and unrelated marrow transplants[1,2]; the significance of pretransplant transfusion-induced sensitization[3]; the requirement of a lymphoid cell population to engraft across DLA barriers[4,5]; and the existence of a radiation-resistant, methotrexate (MTX)-sensitive host cell population which mediates graft rejection in nonsensitized recipients.[6,7]

The present study was designed to further define the morphologic, phenotypic, and functional characteristics of host cells mediating marrow graft resistance in the canine model of unrelated, DLA-nonidentical marrow transplantation, in which there is a 90% graft failure rate.[7] This study was predicated on the hypothesis that the same radioresistant host cells which meidate graft rejection in vivo will inhibit growth of donor marrow in vitro. Therefore, we analyzed recipient marrow and peripheral blood cells at days 3 and 10 after total body irradiation (TBI) and marrow infusion. The results demonstrated a radiation-resitant host lymphoid subpopulation with morphological charcteristics of large granular lymphocytes (LGL), which had natural killer cell (NK) activity and caused suppression of donor marrow growth in vitro.

MATERIALS AND METHODS

Dogs

Beagles and various crossbred hounds were obtained from commercial kennels in Washington and Virginia and through a breeding program at the Fred Hutchinson Cancer Research Center.[8] The dogs weighed 6.0 to 35.5 kg and were 6 to 16 months old (median 12 months) at the time of transplantation. They were dewormed, vaccinated against distemper, leptospirosis, hepatitis, and parvovirus, and housed in single cages.

Selection of Donor-Recipient Pairs

Selection of unrelated, DLA-nonidentical pairs was based upon mutual stimulation of their peripheral blood mononuclear cells in mixed leukocyte culture assay (MLC) and nonidentity of serologically determined DLA antigens.[9] In all cases donor and recipient differed phenotypically for both DLA-D alleles as defined by homozygous cell typing.[9] Sixteen donor-recipient pairs were entered into the study. Ten of sixteen donor-recipient pairs were of different sex.

Total Body Irradiation and Marrow Transplantation

Recipients were prepared for grafting by 9.2 Gy of total body irradiation (TBI) delivered at 7 cGy/minute from two opposing ^{60}Co sources.[10] Recipients were infused with 1.3 to 6.7 × 10^8 (median 3.9 × 10^8, mean 3.98 ± 1.5) marrow cells/kg intravenously within four hours of TBI (day 0). No postgrafting immunosuppression was given and no donor buffy coat cells were infused. Postgrafting care and the marrow aspiration procedure have been described elsewhere.[11,12] Complete blood counts (CBC) were obtained on day 0, before irradiation, and then daily from day 3 postgrafting until death. Cytogenetic analysis of recipient marrow and peripheral blood cells was performed three and ten days after transplantation as described[11] with the only modifications being the use of concanavalin A (Con A, 200 mg/mL Calbiochem-Behring, LaJolla, Calif) to induce mitogenesis. Cultures were harvested on day 3 after exposure to 0.3 μg colchicine for 1½ hours.

Complete autopsies, including marrow specimens for evaluation of cellularity, were performed on ten of 16 recipients.

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Irradiation Controls

Two dogs received TBI without subsequent marrow infusion. The complete blood counts obtained on these dogs and on six irradiation control dogs from a previous study[13] were combined to generate the hematologic data for irradiation controls.

Posttransplant In Vitro studies

In vitro studies were generally performed on days 3 and 10 after transplant.

Donor and recipient marrow and peripheral blood cells. Donor bone marrow was aspirated from the humerus head into a 10-ml syringe containing 5 mL of Waymouth medium (Waymouth-MB752/1, Fred Hutchinson Cancer Research Center media room) and 400 U of preservative-free heparin (Heparin, Sodium, 1,000 USP units/mL, O'Neal, Jones and Feldman, St Louis). Mononuclear marrow cells (MC) were isolated by layering the diluted marrow onto a Ficoll-Hypaque density gradient (1.074 specific gravity), and centrifuging at 1,000 g for 25 minutes Interface cells were washed once with hemolytic buffer, three times with Waymouth medium, and then resuspended in Waymouth medium for cell counting. All washes were at 200 g for ten minutes. Donor peripheral blood mononuclear cells (PBMC) were isolated from venous blood as previously described.[9]

Recipient marrow cells were obtained using a vacuum pump aspiration procedure[12] that permitted the procurement of 50 to 80 mL of marrow mixed with an equal volume of heparinized TC199 medium (GIBCO, Grand Island, NY). This diluted marrow was centrifuged at 500 g for 15 minutes to remove the medium. Red cells were lysed with three hemolytic buffer washes. The resulting cell pellet was washed three times and resuspended in Waymouth medium for cell counts. Viability was determined using trypan blue exclusion. Recipient peripheral blood cells (PBC) were obtained by mixing two parts of heparinized venous blood (from 300 to 600 mL) with one part of Plasmagel (Cellular Products, Buffalo) This mixture was incubated for 45 minutes at 37 °C, 7% CO_2 in air. The resultant supernatants were centrifuged at 500 g for 15 minutes, residual red cells were lysed with a hemolytic buffer wash. The cell pellet was washed three times and resuspended in Waymouth medium for cell counting Obtaining sufficient cell numbers from the recipients for the in vitro studies was a consistent problem necessitating the procurement of large volumes of marrow and peripheral blood.

Morphologic analysis of cells. Cytospin preparations (Cytospin, Shandon-Elliot, Sewickley, Penn) were made of cells obtained on days 3 and 10. These preparations were stained with a standard Wright-Giemsa stain and evaluated for percentage of LGL.

For ultrastructural analysis, cells were fixed in a half-strength Karnofsky's fixative, postfixed in OsO_4 in S collidine buffer and processed for embedding and sectioning in EPON 812 resin, and examined using a JEOL 100-S transmission electron microscope (TEM, Tokyo) as previously described.[14]

Phenotyping of cells with monoclonal antibodies Cells to be phenotyped were incubated with monoclonal antibodies and subsequently analyzed on the fluorescence activated cell sorter (FACS 440, Becton Dickenson FACS System) as previously described.[15] The monoclonal antibodies (McAb) used were 9.6 (IgG_{2b}), which reacts with 99% of human T lymphocytes[16] but does not react with canine PBMC[17], 7.2 (IgG_{2b}), specific for Ia-like antigens on human cells,[18] cross-reactive with an identical antigen (p29/34) on canine cells[17]; DT 2 (IgG_{2a}), which reacts with a large subset of canine T lymphocytes[19]; Dly 6 (IgM), which reacts with virtually all nonactivated canine lymphocytes but not monocytes[20], E11 (IgG_3), which reacts with canine supressor/cytotoxic T lymphocytes[21]; 1A1 (IgM) an anticanine T lymphocyte McAb[22]; F3-20-7 (IgG_1), an anticanine Thy-1 McAb[23]; and SIg, fluorescein-conjugated goat F(ab')$_2$ fragment specific for canine IgG (Cappel, Cochranville, Penn). All of the above monoclonal antibodies with the exception of Dly 6 were directly fluorescein (FITC)-conjugated.[24] The histograms and dot plots generated were used in analysis of these cell populations. A positive phenotyping response was determined by comparing the observed shift of fluorescence intensity of a specific McAb to that obtained with the irrelevant McAb 9.6, and correlating the dot plot data with their respective histograms.

Marrow coculture assays for CFU-GM Donor marrow was cocultured with donor or host cells and the cultures were assayed for the growth of donor marrow colony-forming units–granulocyte/macrophage (CFU-GM), using a modification of an agar-gel medium technique previously described [25,26] The media for marrow cultures consisted of equal volumes of 2× Iscove's medium (GIBCO, Grand Island, NY) and a 0.6% agar (Bacto-Agar, DIFCO, Detroit, Mich) solution in glassed-distilled water. The Iscove's medium was supplemented with eight ribosides (adenosine, guanosine, thymidine, 2-deoxyadenosine, cytidine, uridine, deoxycytidine, 2' deoxyguanosine (Sigma, St Louis), 10 mg each per 500 mL 2 × Iscove's medium). This marrow culture medium also contained 15% fetal calf serum (Irvine Scientific, Santa Ana, Calif) and 5% postendotoxin dog serum (PEDS) for colony-stimulating factor. (PEDS was produced by injecting a dog with 0.5 mg of *Salmonella typhii*, collecting serum three hours later and repeating 1 week later. Sera were pooled, heat-inactivated in a 56 °C water bath, aliquoted, and stored frozen [−20 °C] The marrow culture medium was equilibrated at 39 °C prior to use. 2 × 10^5 donor marrow cells were added to 2.0 mL of marrow culture medium, then either no other cells, donor PBMC, recipient marrow, or peripheral blood cells were added at ratios of 1:1, 1:4, and 1:6, respectively. One mL of these cell mixtures was then added to 35-mm plastic Petri dishes (Falcon #1008, Oxnard, Calif) in duplicate. After the agar solidified at room temperature, the cultures were incubated for ten days in a 100% humidified atmosphere at 37 °C under 7% CO_2 in air. Aggregates of more than 50 cells were counted as colonies using a dissection microscope (Olympus) at a magnification of 25X. As a control, marrow cocultures were also done in 5 donor-recipient pairs prior to TBI and marrow transplantation Results of CFU-GM growth in the various cocultures were compared statistically by nonparametric tests for the comparison of two unpaired samples.[27]

NK assays NK activity of recipient marrow and peripheral blood cells was determined and compared to that of donor marrow and peripheral blood mononuclear cells. A ^{51}Cr (New England Nuclear, Na^{51} CrO, 300 μCi/mL)-labelled canine thyroid adenocarcinoma cell line (CTAC) was used as the target in an 18-hour NK assay using effector-to-target ratios of 20:1 and 50:1, with the results reported as percent specific target cell lysis as previously described.[28]

RESULTS

Peripheral Blood Cell Counts Post-TBI and Marrow Transplantation

Of the sixteen dogs transplanted, two had sustained engraftment and no further studies were done with these two dogs. The other fourteen dogs (88%) failed to engraft and died with aplasia. Figure 1 shows the peripheral white blood cell counts (WBC), including mean absolute granulocyte and mononuclear cell counts, available from eleven recipients. There was a rapid fall in WBC with the mean absolute granulocyte number falling from 8,200 cells/μL pretransplant to 3,000 cells/μL at day 5, to 210 cells/μL at day 7,

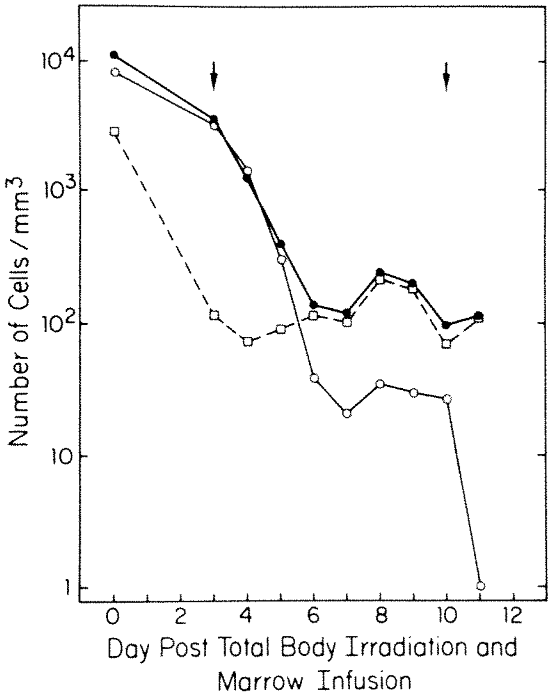

Fig 1. Peripheral white blood cell counts from eleven recipients. The values represent: mean white blood cell counts (●——●); mean absolute granulocyte count (○——○); and mean absolute mononuclear cell count (□----□). The arrows indicate days on which marrow and peripheral blood cells were obtained for in vitro studies. Day 0 was the day of TBI and marrow infusion.

with a rise to 350 cells at day 8 and then decline to zero cells by day 11. However, there was a rise in mean absolute mononuclear cell numbers from a day 3 nadir of 72 cells/μL to 210 cells/μL at day 8 and a final decline to 120 cells/μL by day 11.

Peripheral white blood cell counts from the eight irradiation control dogs are shown for comparison in Fig 2. Similar to the transplanted dogs, there was a rapid fall in the WBC with the mean absolute granulocyte count falling to zero by day 7 post-TBI, with no subsequent rise in counts observed. Of note, the increase in mean absolute mononuclear cell numbers observed from day 3 to day 8 in the transplanted dogs was not seen in the radiation controls. In both transplanted and radiation control dogs, there was a constant decline in mean platelet numbers to less than 1,000/μL at day 10 post irradiation.

Table 1 summarizes the autopsy findings available for ten of the fourteen marrow recipients evaluated in this study. The degree of marrow cellularity in these dogs was expressed as a fraction of normal and ranged from 0% to 10%. In those recipients in whom hemopoietic cell lineage could be evaluated, only focal areas of myeloid, erythroid, or plasma cells were observed.

Cytogenetic Analysis of Recipient Marrow and Peripheral Blood Cells Posttransplant

Cytogenetic analysis on marrow and peripheral blood cells was available in two dogs which failed to achieve sustained engraftment. In both cases only host type cells (19/19 and

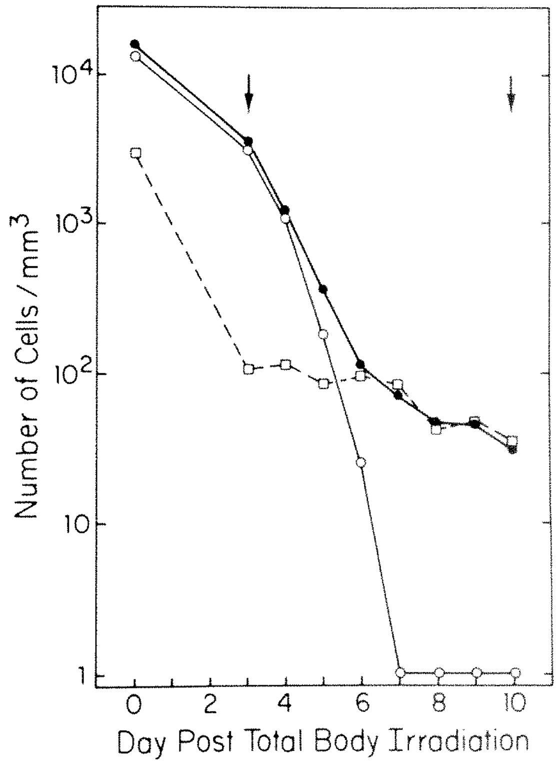

Fig 2. Peripheral white blood cell counts from eight dogs in the radiation control group. The values represent: mean white blood cell count (●——●); mean absolute granulocyte count (○——○); mean absolute mononuclear cell count (□----□). The arrows indicate days on which marrow and peripheral blood cells were obtained for in vitro studies. Day 0 was the day of TBI.

Table 1. Marrow Cellularity and Histopathology of Ten Marrow Recipients at Autopsy

Dog No.*	Survival (days)	Marrow Cellularity (% of normal)	Marrow Cell Lineage	Cause of Death
C34	9	<5%	NA†	Pneumonia; septicemia
C88	10	<5%	NA	Septic shock; pneumonia
C96	4	5% to 10%	Few myeloid cells	Sodium pentothal‡
C99	10	5%	Plasma cells	Sodium pentothal‡
C149	10	0%	NA	Undetermined
C154	10	<10%	Plasma cells	Septicemia
C155	10	0%	NA	Septic shock; pulmonary edema
C157	9	0%	NA	Pneumonia; sodium pentothal‡
C158	10	0%	NA	Undetermined
C208	10	<10%	Erythroid, focal	Hemorrhagic pneumonia

*Complete autopsies were only available on 10 of the 14 recipients evaluated in this study.

†NA, not analyzed for trilineage engraftment due to hypocellularity of marrow.

‡Killed by sodium pentothal injection.

3/3 metaphases, respectively, from Con A–stimulated PBC) were present at day 10 post transplantation. Cytogenetic analysis was hindered by the poor proliferative ability of recipient cells in response to mitogen stimulation. It is possible that the metaphases seen were spontaneous and that the surviving host cells did not possess the capacity to undergo mitogenesis with Con A stimulation.

Morphology of Recipient Peripheral Blood Cells at Days 3 and 10 Posttransplant

Table 2 summarizes the differential cell counts of recipients at three and ten days posttransplantation. LGLs were the predominant lymphoid cells observed on days 3 and 10, 88 ± 9% and 82 ± 13% respectively. Of the total cells on day 3, only 30 ± 27% were LGL, while on day 10, 80 ± 16% were LGL.

Figures 3 and 4 are light and ultrastructural photomicrographs of representative population of LGLs seen in recipient peripheral blood cells at day 10 posttransplant. In Fig 3 the morphological characteristics of LGLs (eg, an eccentric nucleus, relatively large cytoplasmic/nuclear ratio and cytoplasmic granules) are evident. Figure 4 reveals ultrastructural features of canine LGL, including electron dense granules as previously described.[28]

Cytofluorometric Analysis of Recipient Cells

Figure 5 shows representative dot plot profiles of recipient marrow and peripheral blood cells three and ten days posttransplant and of those from the radiation control group at day 10 post-TBI. The population of marrow and peripheral blood cells that showed significant shifts in fluorescence in response to labeling with monoclonal antibodies also showed a forward light scatter characteristic of canine lymphoid cells.[15] On day 3 posttransplant both forward light scatter and the degree of autofluorescence were consistent with the presence of large numbers of granulocytes. This type of profile was also seen with marrow and peripheral blood cells from the radiation control dogs at day 3 post-TBI (dot plots not shown). By day 10, in both recipients and radiation control dogs the cell populations were essentially devoid of granulocytes and highly enriched for lymphoid cells. These findings are consistent with the differential white blood cell counts illustrated in Figs 1 and 2.

Table 2. Differential Cell Counts of Recipient Peripheral Blood Cells Obtained at Days 3 and 10 After Marrow Transplant

Days	Differential† (%)			LGL as % of:‡	
post-BMT*	Lymphocytes	Granulocytes	Monocytes	Lymphocytes	Total
3	34 ± 32	65 ± 32	1 ± 1	88 ± 9	30 ± 27
10	97 ± 4	0 ± 0	3 ± 4	82 ± 13	80 ± 16

*BMT, bone marrow transplant; Day 3 and day 10. Five dogs evaluated.

†Differential on Wright-Giemsa–stained cytospin cell preparations obtained from recipient PBC. Values represent mean ± SEM (%) obtained from eight recipients.

‡LGL, large granular lymphocyte.

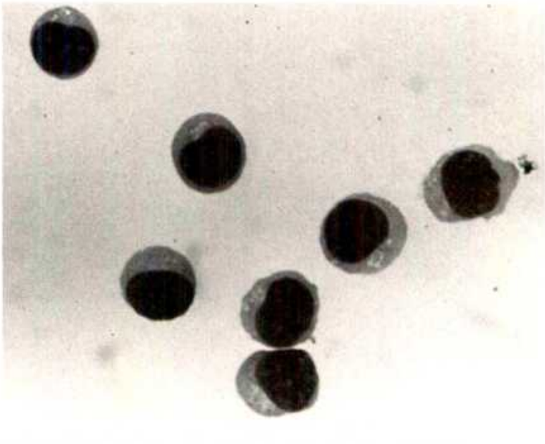

Fig 3. Representative light photomicrograph of large granular lymphocytes (LGL) obtained from recipient peripheral blood on day 10 post transplantation. Characteristics are the cytoplasmic granules located in the perinuclear zone and the eccentric nucleus. Wright-Giemsa stain, original magnification X630; current magnification X315.

On days 3 and 10 posttransplant, recipient marrow and peripheral blood cells were 7.2$^+$, DT2$^+$, 1A1$^+$, Thy 1$^+$, and SIg$^-$. Recipient peripheral blood cells were D1y6$^-$ and E11$^-$ on day 3, but by day 10 posttransplant were D1y6$^+$, and approximately 28% of the peripheral blood cells were E11$^+$. In contrast, the day 3 and 10 marrow cells were D1y6$^+$, with a conversion from E11$^-$ on day 3 to 34% of the marrow cells E11$^+$ by day 10 posttransplant. The percentage of marrow and peripheral blood cells that were found to be positive in their reactivity with E11 was calculated by integrating that portion of the fluorescence peaks obtained from histograms that exceeded the fluorescence obtained using the irrelevant control McAb (9.6). The dot plot patterns for Thy 1$^+$ cells (not shown) from recipient marrow and peripheral blood were comparable to 1A1$^+$. The phenotypes of the radiation

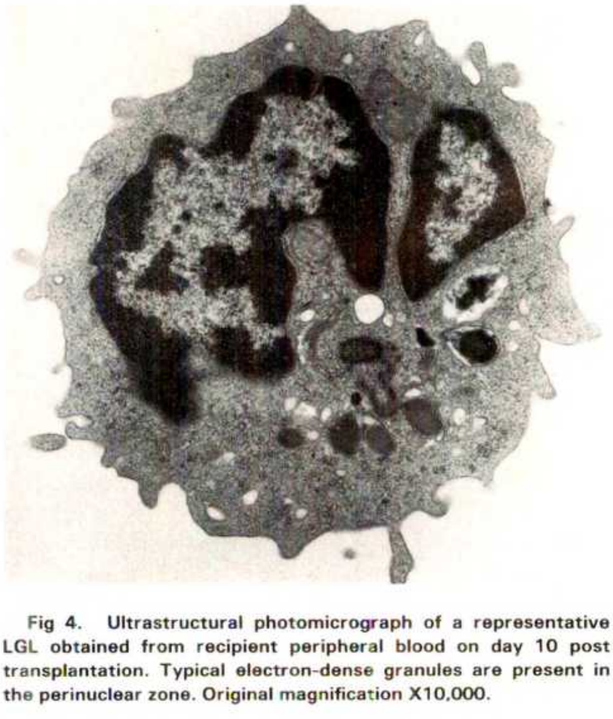

Fig 4. Ultrastructural photomicrograph of a representative LGL obtained from recipient peripheral blood on day 10 post transplantation. Typical electron-dense granules are present in the perinuclear zone. Original magnification X10,000.

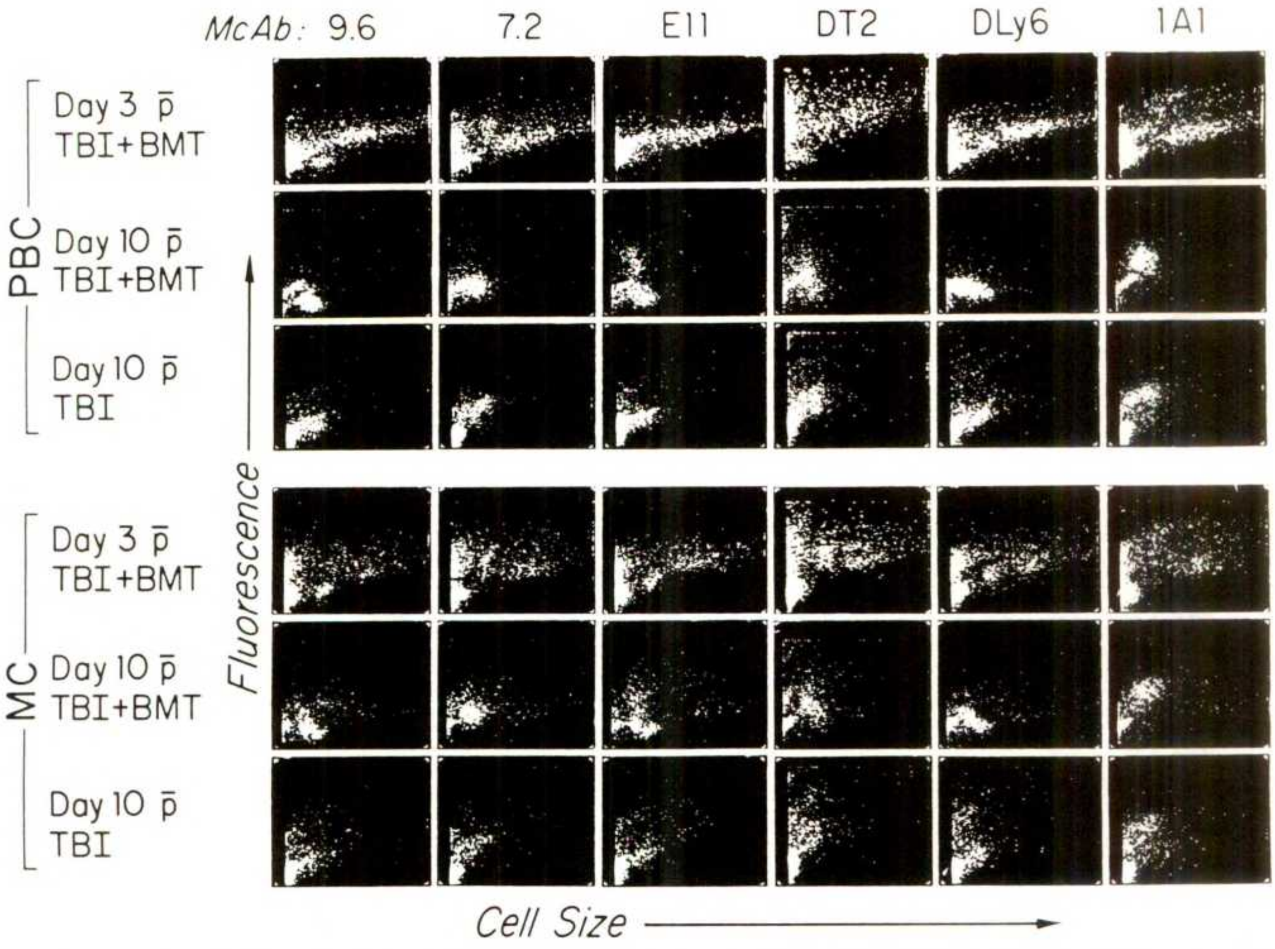

Fig 5. Representative dot plot profiles of recipient marrow (MC) and peripheral blood (PBC) cells on days 3 and 10 post TBI and transplant (BMT), and from the radiation control group on day 10 post TBI. The horizontal axis provides a relative measure of cell size as determined by forward light scatter (smaller cells on the left, larger cells on the right). The vertical axis provides a relative measure of fluorescence intensity. All samples contained approximately 10^6 cells, prepared for cytofluorometric analysis as described under "Materials and Methods."

control dogs' marrow and peripheral blood cells at day 10 post-TBI were 7.2$^+$, DT2$^+$, 1A1$^+$, D1y6$^-$, E11$^-$ and SIg$^-$.

These results suggested that recipient marrow and peripheral blood cells were of lymphoid lineage with the appearance, by day 10, of a subpopulation with the surface antigen phenotype (E11$^+$) previously found to be characteristic for canine suppressor/cytotoxic cells,[21] and also expressed on canine NK cells.[28]

Effects of Recipient Marrow and Peripheral Blood Cells on CFU-GM Growth From Donor Marrow In Vitro

The effect on donor marrow CFU-GM growth by coculturing with donor peripheral blood mononuclear cells, recipient peripheral blood, or recipient marrow cells is illustrated in Fig 6. In all experiments the addition of recipient marrow or peripheral blood cells to donor marrow cells resulted in a decrease in the number of CFU-GM colonies as compared to cultures of donor marrow, either alone or cocultured with donor peripheral blood mononuclear cells. On day 3 post-transplant, the percent inhibition (62% to 67%) of donor marrow CFU-GM growth when cocultured with recipient peripheral blood cells was greater than that obtained from cocultures of recipient marrow cells (49% to 62% inhibition). On day 10, inhibition of donor CFU-GM growth was more profound than on day 3, with cocultures of recipient marrow cells being as potent (70% to 80% inhibition) as peripheral blood cells (62% to 84% inhibition). This increased inhibition coincided with a significant enrichment for large granular lymphocytes by day 10. Cocultures of donor marrow cells with recipient peripheral blood cells obtained before TBI and transplantation did not yield any significant suppression of donor CFU-GM growth.

Similar CFU-GM cultures were carried out using marrow and peripheral blood cells from two dogs in the radiation control group. Cells were obtained on days 3 and 10 after TBI and were cocultured with normal marrow cells from an unrelated, DLA-nonidentical dog. No suppression of CFU-GM growth was observed (data not shown).

NK Activity of Recipient Marrow and Peripheral Blood Cells

Results of NK assays are summarized in Fig 7. Recipient marrow and peripheral blood cells obtained on day 3 post-transplant did not show any detectable NK activity. On day

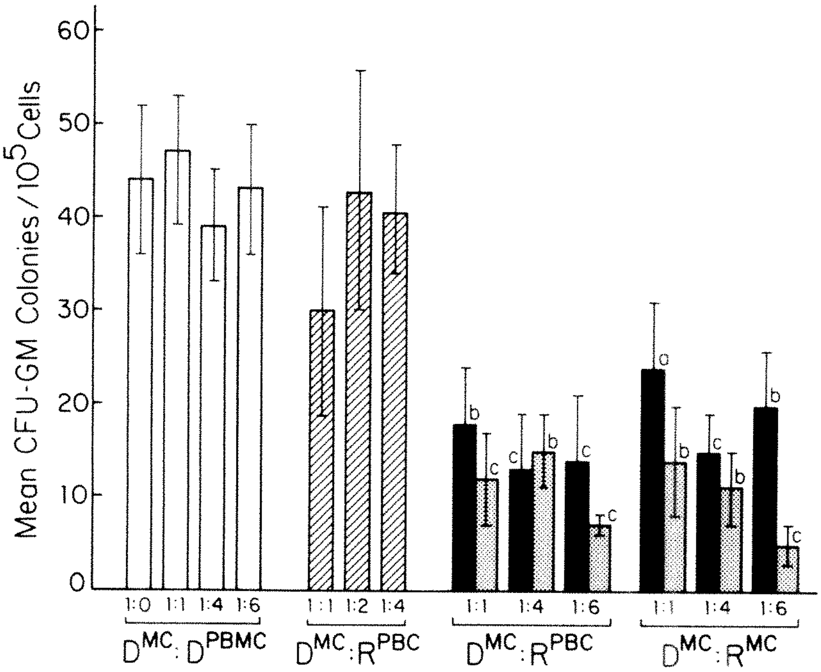

Fig 6. Effect of recipient marrow (R^{MC}) and peripheral blood (R^{PBC}) cells on donor marrow CFU-GM colony growth in vitro. Marrow co-cultures performed on day 3 (■) and on day 10 (░) post transplant. Control cultures were donor marrow (D^{MC}) or donor marrow co-cultured with donor peripheral blood mononuclear cells ($D:D^{PBMC}$) (☐). Co-cultures were carried out at ratios of 1:1, 1:4, and 1:6. Given are the mean number of CFU-GM/10^6 donor marrow cells plated ± SEM (⊢⊣) of eleven experiments. The p values refer to comparisons of CFU-GM growth obtained from D:D co-cultures to D:R co-cultures (a = $p < 0.1$; b = $p < 0.05$; c = $p \le 0.01$). $D^{MC}:R^{PBC}$ co-cultures were performed before TBI and transplantation at ratios of 1:1 and 1:4 (▨).

10 posttransplant NK activity was observed in recipient marrow and peripheral blood cells at the 50:1 effector-to-target ratio (16 ± 5% and 20 ± 8% ^{51}Cr release, respectively).

Marrow and peripheral blood cells obtained from radiation control dogs ten days after TBI also showed NK activity at the 50:1 effector-to-target ratio (59 ± 23% and 21 ± 0% ^{51}Cr release, respectively).

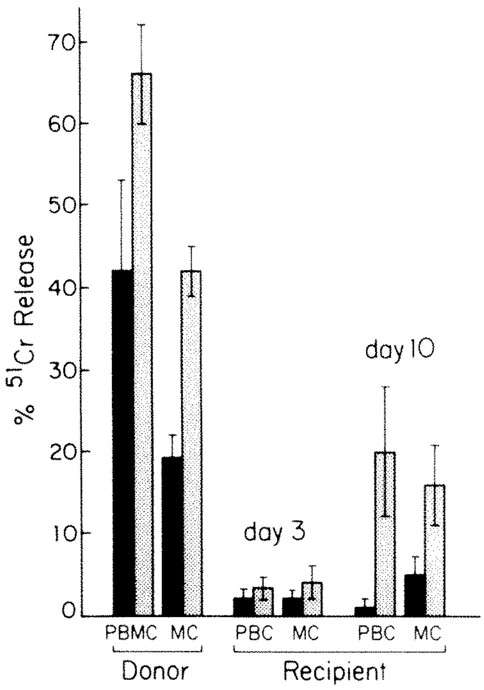

Fig 7. Assays for natural killer (NK) cell activity of recipient marrow (R^{MC}) and peripheral blood (R^{PBC}) cells on days 3 and 10 post transplant and donor marrow (D^{MC}) and peripheral blood mononuclear cells (D^{PBMC}). The effector: target ratios were 20:1 (■) and 50:1 (░). Mean ± SEM % ^{51}Cr release from the CTAC target for four experiments.

DISCUSSION

Previous studies have shown failure of engraftment in 90% of dogs receiving 9.2 Gy of TBI followed by the infusion of DLA-nonidentical donor marrow.[7] The current results (88% failure of engraftment) are in keeping with those data. These and additional studies[1,2,7,29] suggests that in nonsensitized transplant recipients, a relatively radiation-resistant host cell population not requiring pretransplant exposure to donor antigen is involved in mediating resistance to engraftment of DLA-nonidentical marrow. The present study was designed to further characterize these cells.

Host lymphoid cells surviving supralethal doses of TBI were predominantly LGLs capable of suppressing in vitro donor bone marrow cell proliferation as measured by inhibition of CFU-GM growth. It is tempting to speculate that these host cells were responsible for the destruction of the marrow graft in vivo, similar to the mechanisms suggested for allogeneic or hybrid resistance in mice.[30-34] These host cells expressed NK activity when tested in vitro; they had the morphologic appearance previously described for canine NK cells and, except for some minor differences, the phenotype of these LGLs on day 10 posttransplant, Dly 6^+, 1A1$^+$ and E11$^+$ (28% to 34%), was in agreement with that previously described for canine NK cells.[28] Staining with additional monoclonal antibodies recognizing T cell determinants (DT2, Thy 1) suggested a T cell lineage.[19,23]

Of interest was the shift from an E11$^-$ to an E11$^+$ subpopulation in both recipient marrow and peripheral blood cells (34% and 28% positive cells, respectively) between days 3 and 10 posttransplant. Whether this transition indicates a maturation of host LGLs to functional NK cells is conjectural but such a development would be in agreement with the increased NK activity observed on day 10 posttransplant Additional, circumstantial evidence for the NK nature of these cells comes from the lack of Con A–induced mitogenesis observed during cytogenetic analysis of these cells. It has been shown that HNK-1^+ human peripheral blood lymphocytes (NK cells) could not be induced to proliferate when stimulated with various mitogens.[35] Alternatively, the presence of a subpopulation of $E11^+$ lymphoid cells may indicate the differentiation of NK cells into cytotoxic T lymphocytes. Such a mechanism for the activation of NK-derived cytotoxic T lymphocytes regulated by macrophage and prostaglandin has been described in the mouse.[36] Perhaps intact macrophages are involved in the development of NK-derived cytotoxic lymphocytes in the present model in agreement with observations by other investigators of the beneficial effect of macrophage inhibitors on engraftment in recipients of unrelated, DLA-nonidentical marrow.[37] The increase of recipient mononuclear cells observed in the present study approximately eight days posttransplant may indicate such an activation with in vivo proliferation and priming of hosts cells against donor marrow. It is conceivable that a nonprimed (NK type) cell population is operative initially and interferes with engraftment.[30] This may allow for another radiation-resistant host T cell population to be primed and amplified, resulting finally in graft destruction. The transition from $E11^-$ to $E11^+$ host lymphoid cells may signal such an event in the present dog model.

Evidence against the preceding postulated mechanisms is provided by the results of two previous studies which indicated that host cells not of T lymphocyte lineage mediated marrow graft resistance in unrelated DLA-nonidentical transplants.[1,38] In one study, the treatment of recipients with antithymocyte serum alone or in combination with procarbazine before TBI and marrow infusion did not abrogate graft resistance in six of seven recipients.[1] In the second study recipients received cyclosporine after TBI and marrow infusion with eight of 13 recipients failing to show sustained engraftment.[38] These results argue against the involvement of T lymphocytes in marrow graft resistance, but they do not conclusively rule out NK cells or the postulated mechanisms of macrophage-regulated NK-derived cytotoxic T lymphocytes as the effector cells mediating graft resistance. The cells involved in resistance are apparently metabolically active, since treatment of recipients after TBI and marrow infusion with methotrexate abrogates resistance.[6,7]

Recently, resistance to marrow grafts in the mouse was shown to be the result of antibody-dependent cell-mediated cytotoxicity (ADCC), in which NK cells were operating in responder mice in conjunction with a target-specific antibody, resulting in marrow graft destruction.[39] If a similar mechanism is operational in the canine model, in vitro tests of host LGLs carried out early posttransplant might fail to detect significant NK/ADCC-like activity if the appropriate antibody is not present in the culture system. So far no such antibodies have been described in dogs.

The present findings provide additional background data for the design of conditioning regimens capable of overcoming resistance. Preliminary data in the canine model suggest that treatment of recipient dogs with the anti-Ia monoclonal antibody 7.2 in addition to 9.2 Gy of TBI allows for sustained engraftment in a number of dogs.[40] Conceivably, additional more specific monoclonal antibodies (eg 1A1 and E11) or a mixture of antibodies could be even more effective. Whether this information can be applied in clinical marrow transplantation remains to be determined.

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REFERENCES

1. Storb R, Weiden PL, Graham TC, Thomas ED. Failure of engraftment and graft-versus-host disease after canine marrow transplantation. Two phenomena linked to but not exclusively determined by known antigens of the major histocompatibility complex. Transplant Proc 10 113, 1978

2. Deeg HJ, Storb R, Raff RF, Weiden PL, DeRose S, Thomas ED: Marrow grafts between phenotypically DLA-identical and haploidentical unrelated dogs. Additional antigens controlling engraftment are not detected by cell-mediated lympholysis Transplantation 33.17, 1982

3. Storb R, Epstein, RB, Rudolph RH, Thomas ED: The effect of prior transfusion on marrow grafts between histocompatible canine siblings J Immunol 105:627, 1970

4. Storb R, Epstein RB, Bryant J, Ragde H, Thomas ED. Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. Transplantation 6 587, 1968

5. Deeg HJ, Storb R, Weiden PL, Shulman HM, Graham TC, Torok-Storb BJ, Thomas ED. Abrogation of resistance to and enhancement of DLA-nonidentical unrelated marrow grafts in lethally irradiated dogs by thoracic duct lymphocytes. Blood 53:552, 1979

6 Thomas ED, Kasakura S, Cavins JA, Ferrebee JW: Marrow transplants in lethally irradiated dogs. The effect of methotrexate on survival of the host and the homograft. Transplantation 1:571, 1963

7. Weiden PL, Storb R, Graham TC, Sale GE, Thomas ED. Resistance to DLA-nonidentical marrow grafts in lethally irradiated dogs. Transplant Proc 9:285, 1977

8. Ladiges WC, Deeg HJ, Raff RF, Storb R: Immunogenetic aspects of a canine breeding colony. Lab Anim Sci 35.58, 1985

9 Raff RF, Deeg HJ, Farewell VT, DeRose S, Storb R: The canine major histocompatibility complex. Population study of DLA-D alleles using a panel of homozygous typing cells. Tissue Antigens 21:360, 1983

10. Thomas ED, LeBlond R, Graham, T, Storb R: Marrow infusions in dogs given midlethal or lethal irradiation. Radiat Res 41 113, 1970

11. Storb R, Weiden PL, Graham TC, Lerner KG, Nelson N, Thomas ED: Hemopoietic grafts between DLA-identical canine littermates following dimethyl myleran. Evidence for resistance to grafts not associated with DLA and abrogated by antilymphocyte serum. Transplantation 24:349, 1977

12. Kolb HJ, Storb R, Weiden PL, Ochs HD, Kolb H, Graham TC, Floersheim GL, Thomas ED: Immunologic, toxicologic and

marrow transplantation studies in dogs given dimethyl myleran. Biomedicine 20:341, 1974

13 Ochs HD, Storb R, Thomas ED, Kolb HJ, Graham TC, Mickelson E, Parr M, Rudolph RH: Immunologic reactivity in canine marrow graft recipients. J Immunol 113.1039, 1974

14. Brooks CG, Kuribayashi K, Sale GE, Henney CS: Characterization of five cloned murine cell lines showing high cytolytic activity against YAC-1 cells J Immunol 128:2327, 1982

15. Aprile JA, Deeg HJ, Wulff JC, Raff RF· Purified canine monocytes fail to confer concanavalin-A responsiveness to accessory-cell–depleted lymphocytes Exp Hematol 12:45, 1984

16. Kamoun M, Martin PJ, Hansen JA, Brown MA, Siadak AW, Nowinski RC: Identification of a human T lymphocyte surface protein associated with E-rosette receptor. J Exp Med 153·207, 1981

17. Deeg HJ, Wulff JC, DeRose S, Sale GE, Braun M, Brown MA, Springmeyer SC, Martin PJ, Storb R: Unusual distribution of Ia-like antigens on canine lymphocytes. Immunogenetics 16:445, 1982

18. Hansen JA, Martin PJ, Nowinski RC. Monoclonal antibodies identifying a novel T-cell antigen and Ia antigens of human lymphocytes Immunogenetics 10:247, 1980

19. Wulff JC, Deeg HJ, Storb R. A monoclonal antibody (DT-2) recognizing canine T lymphocytes. Transplantation 33:616, 1982

20. Wulff JC, Durkopp N, Aprile J, Tsoi M-S, Springmeyer SC, Deeg HJ, Storb R: Two monoclonal antibodies (Dly-1 and Dly-6) directed against canine lymphocytes Exp Hematol 10:609, 1982

21. Szer J, Deeg HJ, Severns E, Storb R· DLA-D–specific suppressor cells characterized by monoclonal antibodies. Transplantation 39:187, 1985

22. Krawiec DR, Muscoplat CC: Development and characterization of a hybridoma-derived antibody (Aby 1A1) with specificity to canine thymocytes and peripheral T lymphocytes Am J Vet Res 45:491, 1984

23. McKenzie JL, Fabre JW: Studies with a monoclonal antibody on the distribution of Thy-1 in the lymphoid and extracellular connective tissues of the dog. Transplantation 31·275, 1981

24. Clark HF, Shepard CG: A dialysis technique for preparing fluorescent antibody. Virology 20.642, 1963

25. Pike BL, Robinson WA. Human bone marrow colony growth in Agar-gel. J Cell Physiol 76.77, 1970

26. Marsh JC, Levitt M, Katzenstein A: The growth of leukocyte colonies in vitro from dog bone marrow. J Lab Clin Med 79:1041, 1972

27 Tate MW, Clelland RC: Nonparametric and shortcut statistics. Danville, Ill, Interstate Printers, 1957

28. Loughran, TP Jr, Deeg HJ, Storb R. Morphologic and phenotypic analysis of canine natural killer cells: Evidence for T-cell lineage. Cell Immunol 95:207, 1985

29. Deeg HJ, Storb R, Weiden PL, Raff RF, Sale GE, Atkinson K, Graham TC, Thomas ED: Cyclosporin A and methotrexate in canine marrow transplantation: Engraftment, graft-versus-host disease, and induction of tolerance. Transplantation 34:30, 1982

30. Lotzova E, Savary CA: Possible involvement of natural killer cells in bone marrow graft rejection. Biomedicine 27:341, 1977

31. Lotzova E, Savary CA, Pollack SB: Prevention of rejection of allogeneic bone marrow transplants by NK1.1 antiserum. Transplantation 35:490, 1983

32. Lotzova E: Resistance to parental, allogeneic, and xenogeneic hemopoietic grafts in irradiated mice. Exp Hematol 5:215, 1977

33. Kiessling R, Hochman PS, Haller O, Shearer GM, Wigzell H, Cudkowicz G: Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. Eur J Immunol 7.655, 1977

34 Cudkowicz G, Bennett M: Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. J Exp Med 134:83, 1971

35. Abo T, Balch CM: Characterization of HNK-1^+ (Leu-7) human lymphocytes II Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells J Immunol 129·1758, 1982

36 Ting CC, Hargrove ME: Activation of natural killer–derived cytotoxic T lymphocytes 1. Regulation by macrophage and prostaglandins. J Immunol 131:1734, 1983

37. Vriesendorp HM, Klapwijk WM, van Kessel AMC, Zurcher C, van Bekkum DW: Lasting engraftment of histoincompatible bone marrow cells in dogs Transplantation 31:347, 1981

38. Deeg HJ, Storb R, Weiden PL, Graham T, Atkinson K, Thomas ED. Cyclosporin A: Effect on marrow engraftment and graft-versus-host disease in dogs. Transplant Proc 13:402, 1981

39. Warner JF, Dennert G: Bone marrow graft rejection as a function of antibody-directed natural killer cells. J Exp Med 161:563, 1985

40 Deeg HJ, Storb R, Szer J, Appelbaum FR, Hackman RC, Thomas ED: Facilitation of engraftment of DLA-nonidentical marrow by treatment of the recipient with monoclonal anti-Ia antibody. Transplant Proc 17:493, 1985

The Use of the Dilute Russell Viper Venom Time for the Diagnosis of Lupus Anticoagulants

By Perumal Thiagarajan, Vittorio Pengo, and Sandor S. Shapiro

We describe here a test for lupus anticoagulants based on a modified Russell viper venom time (RVVT), using limiting amounts of phospholipid and venom. We have studied 29 patients with a prolonged dilute RVVT. Five of the 29 had a normal activated partial thromboplastin time and three of 14 tested by the tissue thromboplastin inhibition test were normal. In 17 of 19 patients tested, the dilute RVVT was completely normal when ionophore-treated platelets were substituted for phospholipid; the remaining two patients, both with very long phospholipid-dependent dilute RVVT's, were nearly completely normalized. The dilute RVVT is not prolonged in the presence of antibodies to factors VIII, IX, or XI. Thus, the dilute RVVT appears to be a simple, reproducible, sensitive, and relatively specific method for the detection of lupus anticoagulants.

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LUPUS ANTICOAGULANTS are antibodies reactive against anionic phospholipids, thereby prolonging phospholipid-dependent coagulation tests.[1-5] Though initially recognized in patients with systemic lupus erythematosus, similar inhibitors have been described in a variety of disorders and, occasionally, in apparently normal individuals.[2,6-9] In recent years it has become clear that the presence of a lupus anticoagulant is a risk factor for thrombosis[10-18] and recurrent spontaneous abortions.[19-28] Nevertheless, the prevalence of lupus anticoagulants has been difficult to determine, since criteria for the diagnosis of this coagulation inhibitor have not been clearly established. Suspicion of a lupus anticoagulant is usually aroused by the finding of a prolonged activated partial thromboplastin time (aPTT) that is not corrected by addition of an equal volume of normal plasma. Corroboration is frequently sought by performance of the tissue thromboplastin inhibition test (TTI).[9,29] However, the TTI can be negative in the presence of some lupus anticoagulants[30,31] and has been reported to be positive in the presence of some factor VIII or factor IX antibodies, as well as in the presence of heparin.[32] In order to avoid some of these problems, we have employed a modified Russell viper venom time, using diluted venom and a limiting concentration of the phospholipid reagent.[33] We describe here details of the method, as well as some studies of its sensitivity and specificity compared to the TTI and the aPTT in the diagnosis of lupus anticoagulants.

MATERIALS AND METHODS

Coagulation tests. Blood was collected in one-tenth volume of 3.8% trisodium citrate. Platelet-poor plasma was obtained by centrifugation at 2,500 g for 15 minutes at room temperature. The prothrombin time, activated partial thromboplastin time and thrombin time, using tissue thromboplastin (General Diagnostics, Morris Plains, NJ), Thrombofax (Ortho Diagnostic Systems, Raritan, NJ), and Thrombin (Upjohn, Kalamazoo, Mich), respectively, were performed as desribed previously.[34] The normal ranges for these tests in our laboratory are 12 to 15 seconds, 23 to 36 seconds, and 20 to 26 seconds, respectively. The tissue thromboplastin inhibition test (TTI) was performed according to the method of Schleider et al,[9] using a 1:100 dilution of tissue thromboplastin. The titers of antibodies to specific coagulation factors were determined by the Bethesda Assay.[35] All test results described here were performed in our laboratory.

The dilute Russell viper venom time (RVVT) was performed as described previously.[33] Briefly, Russell viper venom (Burroughs Wellcome, Raleigh, NC) was reconstituted as suggested by the manufacturer and further diluted 1:200 in Tris-buffered saline (0.15 mol/L NaCl, 0.02 mol/L Tris, pH 7.5). The phospholipid reagent Thrombofax was diluted 1:8 in Tris-buffered saline. Other partial thromboplastins can be substituted for Thrombofax, however, the dilution of each reagent needs to be determined in the manner described for Thrombofax (see Results). The dilute RVVT was performed by incubating 0.1 mL of plasma, 0.1 mL of diluted Russell viper venom, and 0.1 mL diluted phospholipid for 30 seconds at 37 °C, after which 0.1 mL of 0.03 mol/L calcium chloride was added and the clotting time recorded. In experiments using platelets, 0.1 mL of calcium ionophore–treated platelets was substituted for phospholipid.

Preparation of washed, ionophore-treated platelets. Citrated blood was centrifuged at 150 g for 15 minutes at room temperature. The platelet-rich plasma supernatant was centrifuged at 1,500 g for ten minutes at room temperature and the platelets were washed twice by suspension and centrifugation in a buffer consisting of 0.15 mol/L NaCl, 0.02 mol/L Tris, 0.001 mol/L EDTA, 0.005 mol/L glucose, pH 7.5. The twice-washed platelets were resuspended at a concentration of 8×10^8/mL in the same buffer, but without EDTA. Activation was achieved by adding to 2 mL of platelet suspension 1 µL of a 5 mmol/L solution of calcium ionophore A23187 (Calbiochem, San Diego, Calif) in absolute ethanol (giving a final concentration of 2.5 µmol/L, and incubating for five minutes at room temperature. The ionophore-treated platelets were used immediately or frozen in aliquots at −70 °C for future use.

RESULTS

Effect of phospholipid concentration on the RVVT. Russell viper venom was diluted to give a clotting time in normal plasma of approximately 25 seconds. At this dilution, slightly better separation of lupus anticoagulant and normal plasma was achieved than at higher venom concentrations. Since lupus anticoagulants are antibodies with immunologic reactiv-

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ity towards anionic phospholipids, we wished to increase the test sensitivity further by using the minimal concentration of phospholipid reagent necessary for prothrombin activation. As can be seen in Fig 1, Thrombofax was slightly inhibitory when used undiluted, and was optimal at a dilution of 1:2 to 1:4, when tested in normal plasma. However, optimal separation of lupus anticoagulant from normal plasma was achieved at a Thrombofax dilution of 1:8 or greater. At this phospholipid concentration lupus anticoagulant patients were clearly abnormal, even though some had a normal RVVT with undiluted phospholipid reagent. The coefficient of variation of this test, determined by performing 20 replicates on a single normal plasma, was 0.8%. The normal range, determined on 14 fresh normal plasmas, was 26.2 ± 1.5 sec (± 2 SD). The normal range observed in our laboratory over a 3-year period, using fresh and frozen plasmas, was 25.6 ± 2.6 sec (± 2 SD). We consider clotting times of 30 seconds or greater (>3.8 SD above the mean) to be abnormal, and make a presumptive diagnosis of a lupus anticoagulant when an abnormal test is not corrected by addition of an equal volume of normal plasma Using this test, we have diagnosed the presence of a lupus anticoagulant in 29 patients referred to us because of the suspicion of such an anticoagulant. Of the 29 patients, only 24 had a prolonged aPTT. Of the remaining five, two patients were on steroid therapy at the time of our study but had a prolonged aPTT previously; one patient had a chloropromazine-related lupus syndrome with an antinuclear antibody titer of 1:320 and a rapid plasma reagin (RPR) titer of 1:16; the fourth patient had a history of recurrent abortions; and the fifth patient had systemic lupus erythematosus.

Substitution of platelets for phospholipid in the dilute RVVT. The effect of substituting calcium ionophore-activated platelets for the phospholipid reagent was investigated as follows. Ionophore-treated platelets were diluted to a concentration giving an RVVT of 23 to 28 seconds. The concentration of platelets necessary to give this clotting time is variable with the platelet preparation, ranging between 50 × 10^8/mL and 200 × 10^8/mL. Washed, ionophore-treated platelets are stable in suspension at least during the day they are prepared, and can be quick-frozen at least once and stored for 12 months or more at −70 °C without loss of activity. As can be seen in Fig 2, in 17 of 19 cases tested in this manner the RVVT was completely normal when performed with platelets rather than phospholipid. In the other two cases the RVVT was not completely normalized. These two patients had very prolonged dilute RVVTs and, in addition, one of the two was receiving oral anticoagulants.

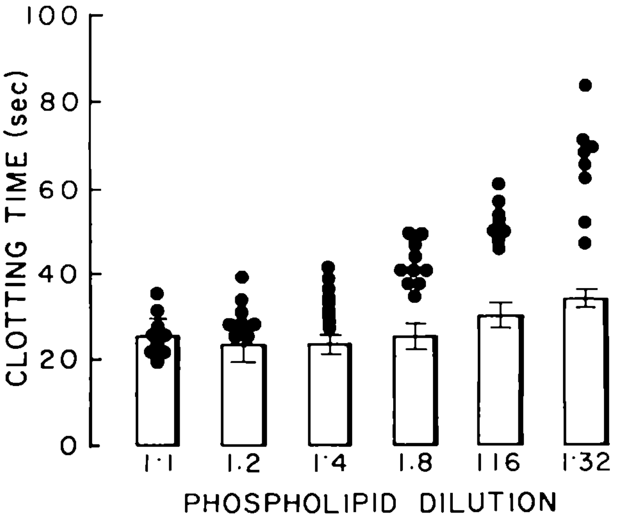

Fig 1. Effect of phospholipid concentration on the dilute RVVT. Bars are normal controls and closed circles are lupus patients.

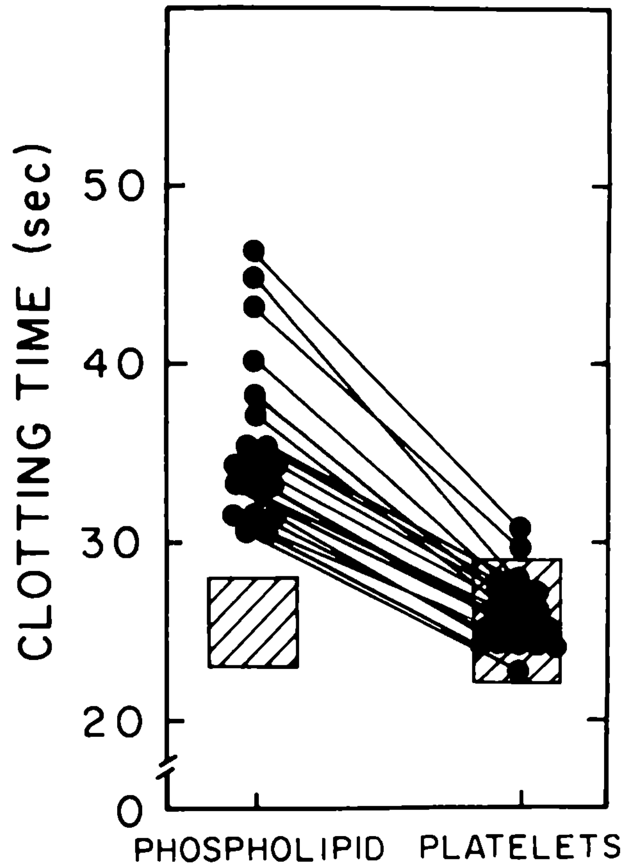

Fig 2. Effect of platelets and phospholipid on the RVVT of lupus anticoagulant plasmas. Hatched areas are the 2 SD normal ranges.

The dilute RVVT in coagulation factor deficiencies. The dilute RVVT is normal in plasma deficient in factors VII, VIII, IX, XI, or XII. Plasmas with factor V or factor X levels below 0.4 U/mL and plasmas from patients receiving oral anticoagulants give a prolonged dilute RVVT. However, in these situations the prolonged test is normalized by the addition of an equal volume of normal plasma (Fig 3). In contrast, addition of an equal volume of normal plasma does not correct the dilute RVVT of lupus anticoagulant plasma. In fact, an occasional lupus anticoagulant plasma shows a further prolongation of the RVVT test on admixture with normal plasma (Fig 3), an effect previously referred to as the "lupus cofactor" phenomenon.[36,37]

The dilute RVVT in the presence of heparin or antibodies to coagulation factors. The presence of antibodies to factors VIII, IX, or XI does not prolong the dilute RVVT (Table

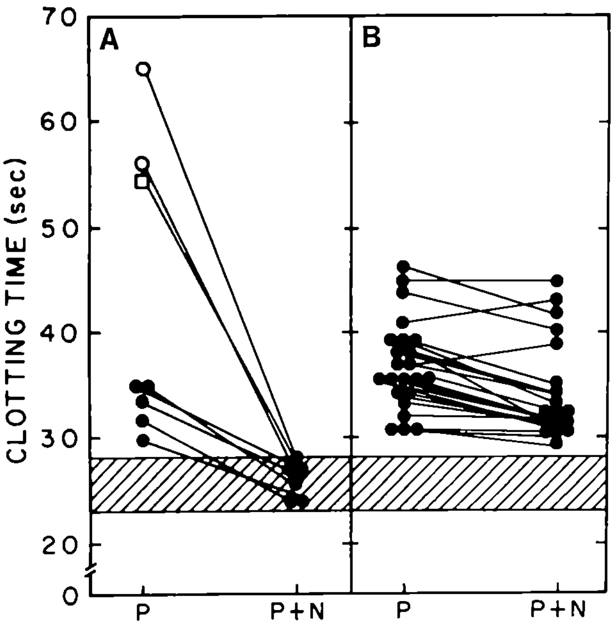

Fig 3. (A) Effect of normal plasma on the dilute RVVT of factor-deficient plasmas. O, factor X-deficient plasma; □, factor V-deficient plasma; ●, plasma of patients on oral anticoagulants. (B) Effect of normal plasma on the dilute RVVT of lupus anticoagulant plasma. Equal volumes of patient (P) and normal (N) plasma were mixed and the dilute RVVT recorded. Hatched areas are the 2 SD normal range.

1). The presence of an antibody to factor V does prolong the test. However, in contrast to the lupus anticoagulant, the prolonged dilute RVVT of factor V antibody plasma does not correct when ionophore-activated platelets are used (Table 2).

As shown in Fig 4, the dilute RVVT, like the aPTT, is prolonged by therapeutic levels of heparin. The dilute RVVT appears to be less sensitive than the aPTT in this regard. Below 0.2 U/mL of heparin, the prolongation of the dilute RVVT is corrected when ionophore-treated platelets are substituted for phospholipid (data not shown).

Comparison with the aPTT and the tissue thromboplastin inhibition test. As can be seen in Fig 5A, 25 of 29 patients had prolongations of the aPTT and the dilute RVVT, while four patients with an abnormal dilute RVVT had a normal aPTT. As shown in Fig 5B, 11 of 14 patients studied were abnormal in both dilute RVVT and TTI tests. However, two patients with potent IgM lupus anticoagulants had a normal TTI while a third patient, with a weak lupus anticoagulant, was also normal in the TTI test. Thus, although the correlation between results of all three tests was good, the dilute RVVT appears to be the most sensitive of the three.

Table 1. Effect of Antibodies to Coagulation Factors on the Dilute RVVT

Antibody	Titer (Bethesda U/mL)	Dilute RVVT (seconds)
Factor VIII	38	25.9
Factor VIII	87	26.3
Factor VIII	7	26.8
Factor VIII	23	25.7
Factor VIII	14	26.7
Factor IX	85	27.1
Factor XI	415	26.4

Table 2. Effect of Factor V Antibody on the Dilute RVVT

Factor V Antibody Concentration (Bethesda U/mL)	Dilute RVVT Phospholipid (seconds)	Dilute RVVT Platelets (seconds)
663.0	68.9	59.2
66.3	66.9	57.8
6.63	61.9	56.7
0.663	38.5	40.1
0.066	32.3	29.2
0	25.4	26.9

DISCUSSION

A number of different tests have been proposed and used for the diagnosis of lupus anticoagulants, reflecting the difficulty in reaching a consensus concerning their definition and mechanism of action.[9,38,39,40] It is now clear that lupus anticoagulants have immunologic reactivity towards anionic phospholipids and thereby prolong phospholipid-dependent coagulation tests. Nevertheless, phospholipid-dependent tests may show variable sensitivity and/or specificity toward lupus anticoagulants for several reasons. First, there are no standards for phospholipid reagents used in coagulation tests, and the quantity of phosphatidylserine present in such reagents, for example, has been shown to affect their sensitivity toward lupus anticoagulants.[41,42] One test[38] does not utilize any added phospholipid, presumably relying on phospholipid originating from the plasma or the "platelet dust."[43,44] Second, the physical state of the phospholipid differs in different tests. the aPTT and the RVVT utilize

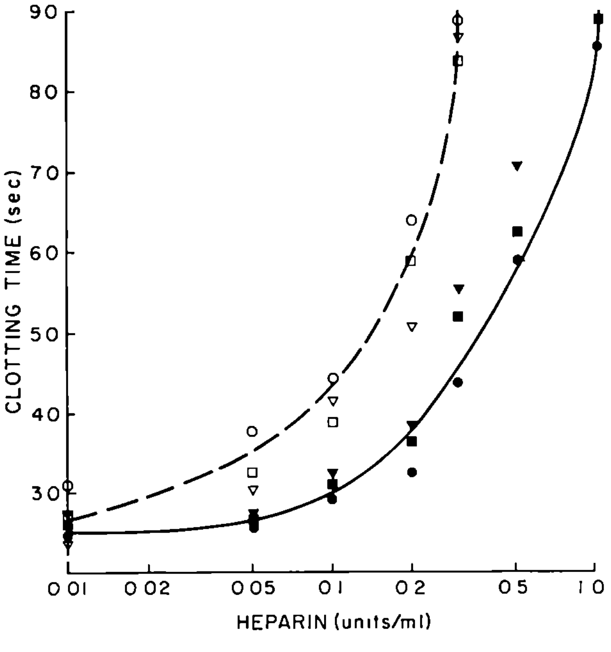

Fig 4. Effect of heparin on the aPTT and the dilute RVVT of three normal plasmas. Open symbols, aPTT; closed symbols, dilute RVVT.

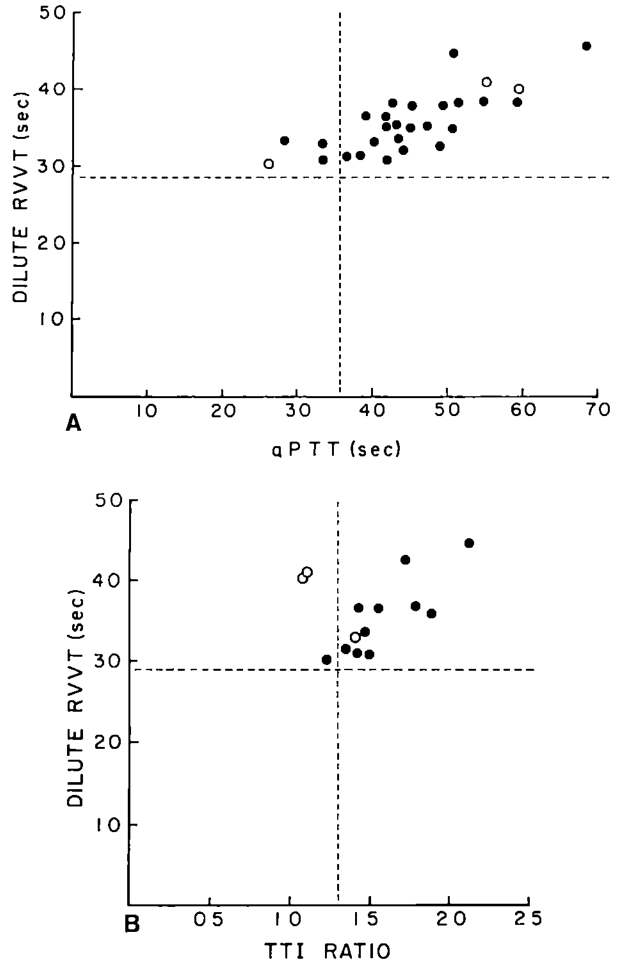

Fig 5. Comparison of the dilute RVVT with the aPTT (A) and the TTI (B). Open circles are patients with IgM lupus anticoagulants. Closed circles are patients with IgG or a mixture of IgG and IgM lupus anticoagulants. Dotted lines indicate upper limits of normal for the tests.

phospholipid in micellar form, whereas the TTI and the prothrombin time (PT) make use of tissue thromboplastin, in which the phospholipid is protein-bound. Third, the tests vary in their response to deficiencies of clotting factors and to antibodies to clotting factors. For instance, antibodies to factor VIII may prolong the aPTT, but not the RVVT. Finally, there appear to be variations in test sensitivity related to the immunoglobulin class of the lupus anticoagulant; for example, as we have shown, some IgM anticoagulants do not prolong the TTI, although they do prolong other tests. As a result, the incidence of lupus anticoagulants has not been clearly established, since patients may be positive in one test and not another.

Strategies to increase the sensitivity of tests have taken two forms. Since lupus anticoagulant activity is less evident in the presence of platelets,[1,3,45,46] the use of platelet-free plasma has been advocated. Second, as in our procedure, several tests make use of limiting concentrations of phospholipids to increase their sensitivity. We have chosen the RVVT as our primary test for the diagnosis of lupus anticoagulants, since it is unaffected by the presence of antibodies to factors VIII, IX, or XI, and have further increased the sensitivity of the test by using limiting concentrations of both phospholipid and venom. This test is easy to perform and highly reproducible. Nevertheless, it is not entirely specific for lupus anticoagulants. Coagulation factor deficiencies can prolong the dilute RVVT, but these can be easily excluded by repeating the test on a mixture of normal and patient plasma. The presence of an antibody to factor V, an extremely rare event, also prolongs this test. However, in one factor V antibody patient whom we had the opportunity to study, substitution of ionophore-treated platelets for the phospholipid reagent did not correct the test, unlike the findings with lupus anticoagulants. Furthermore, the presence of a factor V antibody is associated with a marked prolongation of the PT, a rare occurrence with lupus anticoagulants. Nevertheless, definitive exclusion of a factor V antibody requires specific measurement of factor V inhibition in a mixture of patient and normal plasma.

Finally, therapeutic concentrations of heparin prolong the dilute RVVT, although to a somewhat lesser extent than the aPTT (Fig 4). Heparin effects can be ruled out by measurement of the thrombin time, since this test is normal in the presence of a lupus anticoagulant.

The significance of the presence of a lupus anticoagulant does not lie in its association with bleeding, which rarely, if ever, occurs as a result of the lupus anticoagulant alone,[3,7,8,15] but rather in the fact that approximately 25% to 30% of patients with this antibody have a history of thromboembolic phenomena,[10–18] or repeated spontaneous abortions.[14–28] The presence of anticardiolipin antibodies, as measured by any one of several immunologic techniques, has also been identified as a risk factor for thrombosis.[5,24,47] However, the relationship between these two measurements has not been evaluated adequately. It is clear, for example, that some types of "anticardiolipin" antibodies are not associated with an increased thrombotic risk. For example, positive serologic tests in patients with syphilis are due to reactivity of patient serum with cardiolipin, yet patients with syphilis do not have an increased prevalence of lupus anticoagulants or of thromboembolic disease.[48] For an adequate understanding of the biologic role of antibodies reactive with anionic phospholipids, it will be necessary to standardize both types of tests. The dilute RVVT which we describe here is simple and reproducible. Moreover, as we have demonstrated, it is more sensitive than the aPTT and the TTI, and thus should be highly useful as a screening diagnostic test for further studies.

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REFERENCES

1 Thiagarajan P, Shapiro SS, DeMarco L A monoclonal IgM lambda coagulation inhibitor with phospholipid specificity: Mechanism of a lupus anticoagulant. J Clin Invest 66:397, 1980

2. Shapiro SS, Thiagarajan P, DeMarco L: Mechanism of action of the lupus anticoagulant. Ann NY Acad Sci 370.359, 1981

3 Shapiro SS, Thiagarajan P: Lupus anticoagulants. Prog Thromb Hemost 5:2163, 1982

4. Lafer EM, Rauch J, Andrzsejewski C, Mudd D, Furie B, Schwartz RS, Stollar BD: Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. J Exp Med 153:897, 1981

5. Harris EN, Gharavi AE, Tincani A, Chan JKH, Englert H, Mantelli P, Allegro F, Ballestrieri G, Hughes GRV· Affinity purified anticardiolipin and anti-DNA antibodies. J Clin Lab Immunol 17·155, 1985

6 Manoharan A, Gibson L, Rush B, Feery BJ: Recurrent venous thrombosis with a lupus coagulation inhibitor in the absence of systemic lupus. Aust NZ J Med 7 422, 1977

7. Feinstein DI, Rapaport SI Acquired inhibitors of blood coagulation. Prog Thromb Haemost 1:75, 1972

8. Lechner K: Acquired inhibitors in non-hemophiliac patients Haemostasis 3:65, 1974

9. Schleider MA, Nachman RL, Jaffe EA, Coleman M. A clinical study of the lupus anticoagulant. Blood 48:499, 1976

10. Bowie EJW, Thompson JH Jr, Pascuzzi CA, Owen CA Jr: Thrombosis in systemic lupus erythematosus despite circulating anticoagulants J Lab Clin Med 62:416, 1963

11. Carreras LO, Vermylen JG: Lupus anticoagulant and thrombosis—Possible role of inhibition of prostacyclin formation. Thromb Haemost 48.38, 1982

12 Carreras LO, Defreyn G, Machin SJ, Vermylen J, Deman R, Spitz B, Assche AV: Arterial thrombosis, intrauterine death and "lupus" anticoagulant. Detection of immunoglobulin interfering with prostacyclin formation Lancet 1 244, 1981

13. Boey ML, Colaco CB, Gharvai AE, Elkon KB, Loizou S, Hughes GRV· Thrombosis in systemic lupus erythematosus: Striking association with the presence of circulating lupus anticoagulant. Br Med J 287 1021, 1983

14 Williams H, Laurent R, Gibson T: Lupus coagulation inhibitor and thrombosis: Report of 4 cases. Clin Lab Haematol 2:139, 1980

15. Glueck HI, Kant KS, Weiss MA, Pollack VE, Miller MA, Coots M· Thrombosis in systemic lupus erythematosus: Relation to the presence of circulating anticoagulant. Arch Intern Med 145:1389, 1985

16. Mueh JR, Herbst KD, Rapaport SI: Thrombosis in patients with the lupus anticoagulant. Ann Intern Med 92:156, 1980

17. Elias M, Eldor A: Thromboembolism in patients with the "lupus"-type circulating anticoagulant. Arch Intern Med 144:510, 1984

18. Lechner K, Pabinger-Fasching I: Lupus anticoagulant and thrombosis: A study of 25 cases and review of literature. Haemostasis 15 254, 1985

19. Nilsson IM, Astedt B, Hedner U, Berezin D: Intrauterine death and circulating anticoagulant ("antithromboplastin") Acta Med Scand 197.153, 1975

20 Farquharson RG, Pearson JF, John L: Lupus anticoagulant and pregnancy management. Lancet 2:228, 1984

21. Gardlund B: The lupus inhibitor in thromboembolic disease and intrauterine death in the absence of systemic lupus. Acta Med Scand 215:293, 1984

22. Lubbe WF, Liggins GC: Lupus anticoagulant and pregnancy Am J Obstet Gynecol 153:322, 1985

23. Reece EA, Romero R, Clyne LP, Kriz NS, Hobbins JC: Lupus-like anticoagulant in pregnancy. Lancet 1.344, 1984

24. Lockshin MD, Duzin ML, Goeli S, Qamar T, Magid MS, Jovanovic L, Ferenc M: Antibody to cardiolipin as a predictor of fetal distress or death in pregnant patients with systemic lupus erythematosus. N Engl J Med 313:152, 1985

25. Firkin BG, Howard MA, Radford N: Possible relationship between lupus inhibitor and recurrent abortion in young women. Lancet 2:366, 1980

26. Gabriel L, Samama M, Conard J, Horellou MH, Servelle M: Anticoagulant circulant antiprothombinase, thromboses et avortements spontaneous, une nouvelle observation. Nouv Presse Med 9.2159, 1980

27 Soulier JP, Boffa MC: Avortements a repetition, thromboses et anticoagulant circulant anti-thromboplastine· trois observations. Nouv Presse Med 9.859, 1980

28. De Wolf F, Carreras LO, Moerman P, Vermylen J, van Assche A, Renaer M: Decidual vasculopathy and extensive placental infarction in a patient with repeated thromboembolic accidents, recurrent fetal loss and a lupus anticoagulant Am J Obstet Gynecol 142:829, 1982

29. Boxer M, Ellman L, Carvalho A: The lupus anticoagulant. Arthritis Rheum 19·1244, 1976

30. Canoso RT, Hutton RA, Deykin D. A chloropromazine induced inhibitor of blood coagulation. Am J Hematol 2 183, 1977

31. Canoso RT, Sise HS. Chloropromazine-induced lupus anticoagulant and associated immunologic abnormalities. Am J Hematol 13:121, 1982

32. Triplett DA, Brandt JT, Kaczor D, Schaeffer J: Laboratory diagnosis of lupus inhibitors: A comparison of the tissue thromboplastin inhibition procedure with a new platelet neutralization procedure Am J Clin Pathol 79·678, 1983

33. Thiagarajan P, Shapiro SS: Lupus anticoagulants. In Colman RW (ed): Methods in Hematology. Disorders of thrombin formation other than hemophilia. Livingston, New York, 1983, p 101

34. Tocantins LM, Kazal LA· Blood coagulation. Hemorrhage and Thrombosis. Grune & Stratton, Orlando, Fl, 1964, p ?

35. Kasper CK, Aledort LM, Counts RB, Edson JR, Fratantoni J, Green D, Hampton JW, Hilgartner MW, Lazerson J, Levine PH, McMillan CW, Pool JG, Shapiro SS, Shulman NR, van Eys J. A more uniform measurement of factor VIII inhibitors. Thromb Diath Haemorrh 34:869–872, 1975

36. Loeliger A: Prothrombin as cofactor of the circulating anticoagulant in systemic lupus erythematosus? Thromb Diath Haemorrh 3.237, 1959

37. Rivard GE, Schiffman S, Rapaport SI: Cofactor of the lupus anticoagulant. Thromb Diath Haemorrh 32:554, 1974

38. Exner T, Rickard KA, Kronenberg H. A sensitive test demonstrating lupus anticoagulant and its behavioural pattern. Br J Haematol 40:143, 1978

39. Alving BM, Baldwin PE, Richards RL, Jackson BJ: The dilute phospholipid APTT· A sensitive assay for verfication of lupus anticoagulants. Thromb Haemost 54:709, 1985

40. Green D, Hougie C, Kazmier FJ, Lechner K, Mannucci PM, Rizza CR, Sultan Y· Report of the working party on acquired inhibitors of coagulation· Studies of the "lupus" anticoagulant. Thromb Haemost 49:144, 1983

41. Kelsey R, Stevenson KJ, Poller L· The diagnosis of lupus anticoagulants by the activated partial thromboplastin time. The central role of phosphatidylserine. Thromb Haemostas 52:172, 1984

42 Mannucci PM, Canciani MT, Mari D, Meucci P. The varied sensitivity of partial thromboplastin and prothrombin time reagents in the demonstration of lupus-like anticoagulants Scand J Haematol 22:423, 1979

43 Boffa MC: Evaluation of the phospholipid-related procoagulant activity in plasma A new clue for detecting tendency of thrombosis. Thromb Res 17.567, 1980

44 Crawford N: The presence of contractile proteins in platelet microparticles isolated from human and animal platelet-free plasma. Br J Haematol 21.53, 1971

45 Firkin BG, Booth P, Hendrix L, Howard MA· Demonstration

of a platelet by-pass mechanism in the clotting system using an acquired anticoagulant. Am J Hematol 5:81, 1978

46. Howard MA, Firkin BG: Investigations of the lupus-like inhibitor by-passing activity of platelets. Thromb Haemost 50:775, 1983

47. Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GRV: Anti-cardiolipin antibodies: Detection by radioimmunoassay and association with thrombosis in SLE. Lancet 2:1211, 1983

48. Johannson EA, Lassus A. The occurrence of circulating anticoagulants in patients with syphilitic and biologically false positive antilipoidal antibodies Ann Clin Res 6:105, 1974

Complement Proteins C5b-9 Stimulate Procoagulant Activity Through Platelet Prothrombinase

By Therese Wiedmer, Charles T. Esmon, and Peter J. Sims

The capacity of platelets treated with nonlytic concentrations of the C5b-9 proteins to catalyze prothrombin activation and thereby trigger clot formation has been investigated. When suspended in the presence of exogenous factors Xa and Va, gel-filtered platelets treated with purified C5b-9 proteins catalyzed prothrombin to thrombin conversion at rates up to tenfold above controls, and exceeded by up to fourfold the prothrombinase activity observed for thrombin-stimulated platelets. In the absence of added factor Va, C5b-9 assembly on the platelet surface significantly shortened the lag period before prothrombinase expression that was observed for untreated platelets and increased the maximum catalytic rate of thrombin formation. A comparison with other platelet stimuli revealed that the C5b-9-induced activation of platelet prothrombinase closely paralleled the effects mediated by calcium ionophore A23187. Our data suggest that the C5b-9 proteins promote the release of platelet factor V and the assembly of the prothrombinase complex, thereby potentiating the effects of thrombin on the activation of prothrombinase. Membrane assembly of the C5b-9 proteins was also observed to markedly accelerate the rate of platelet-catalyzed plasma clotting, suggesting a direct link between C5b-9-mediated prothrombinase activation and procoagulant activity accompanying immunologic damage to the platelet.

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THERE IS NOW substantial evidence that the normal hemostatic function of the blood platelet can be affected by various components of the complement system.[1] Exposure of platelet-rich plasma to zymosan, an activator of the alternative complement pathway, results in platelet aggregation and release,[2] as well as increased platelet procoagulant activity.[3] Using gel-filtered platelets, Polley and Nachman[4,5] observed that thrombin-stimulated aggregation and serotonin release are potentiated in the presence of C3 plus C5 through C9, and they presented evidence that this increased response is directly related to thrombin-initiated assembly of C5b-9, which occurs without lysis of the plasma membrane. The potential for sublytic modulation of platelet function by the terminal complement proteins has also been described by Hansch et al,[6] who reported that C5b-9 binding to the platelet surface can stimulate arachidonate metabolism through the cyclooxygenase pathway. We have recently reported that the purified C5b-9 proteins, when assembled on gel-filtered platelets, can modulate the electrochemical state of the plasma membrane in the absence of cell lysis, initiating a transient and reversible depolarization of membrane potential.[7] Furthermore, insertion of the C5b-9 proteins into the platelet plasma membrane results in a rise in cytoplasmic Ca^{2+}[8] and vesiculation of the plasma membrane with concomitant removal of the C5b-9 complex,[9] raising the potential role of terminal complement proteins in the modulation of membrane-localized reactions of the coagulation pathway. We now demonstrate directly that assembly of the purified complement proteins C5b-9 on the plasma membrane of gel-filtered platelets increases platelet prothrombinase activity without lysing the cell, and that this cellular response can be triggered in the absence of exogenous factor Va.

MATERIALS AND METHODS

Materials. Prostaglandin E_1 (PGE_1), nystatin, and bovine serum albumin (fatty acid–free) were purchased from Sigma, St Louis, and A23187 was obtained from Calbiochem, San Diego Spectrozyme TH was from American Diagnostica, Greenwich, Conn. All other chemicals were of reagent or analytical grade.

Solutions. Medium I. 145mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 0.1% (wt/vol) bovine serum albumin, 25 nmol/L PGE_1, and 5 mmol/L PIPES, pH 6.8. Medium II· 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 1% (wt/vol) bovine serum albumin, and 5 mmol/L HEPES, pH 7.4. Medium III: Medium II containing 2.5 mmol/L $CaCl_2$.

Platelets. Freshly prepared platelet-rich plasma from normal volunteers was obtained through the facilities of the Oklahoma Blood Institute PGE_1 was added at a final concentration of 25 nmol/L. Except where indicated, platelets were kept at room temperature and plastic was used throughout. After an initial low-speed spin to remove contaminating red cells (four minutes, 200 g), the platelets were concentrated by centrifugation (20 minutes, 500g) After careful suspension in 0.5 to 1 mL of Medium I, the platelets were gel-filtered on a column (0.9 × 30 cm) of Sepharose CL-2B (Pharmacia, Piscataway, NJ) equilibrated in Medium I. Cell counting and resistive sizing were performed using Models ZBI and Channelyzer (C 1000), Coulter Electronics, Hialeah, FL.

Coagulation proteins. Bovine factor Va,[10] factor Xa,[11] prothrombin[11] and human and bovine thrombin[11] were isolated by minor modifications[12] of the methods cited above.

Complement proteins. Human complement proteins C5b6, C7, C8 and C9 were purified and analyzed for functional activity as previously described.[13-16] Before addition to platelet suspensions, the proteins were dialyzed into Medium II.

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C8-deficient plasma. Citrated plasma was depleted of C8 by absorption against the IgG fraction of goat antiserum raised against purified C8.

C5b67 platelets. To assemble membrane-bound C5b67 complexes, gel-filtered platelets were suspended with C5b6 and C7 (15 μg and 5 μg, respectively, per 1 × 10^8 cells) with rapid mixing in a final volume of 100 μl. After incubation for three minutes at 37 °C the C5b67 platelets were recovered and used immediately.

C5b-9 platelets To assemble membrane-bound C5b-9, C5b67 platelets were suspended in Medium III at 1 × 10^8 cells/mL and incubated for 10 minutes at 37 °C in presence of 4 μg/mL of C9 and various amounts of C8 as indicated in the text.

Platelet prothrombinase. Gel-filtered platelets (untreated, or treated with complement proteins, thrombin, or ionophores) were suspended at 37 °C in Medium III at 8 × 10^7/mL in the presence of 2.7 μmol/L prothrombin, and, either 0 or 2 nmol/L factor Va. The reaction was started by addition of factor Xa (final concentration 1 nmol/L), and at the times indicated in figure legends, 50-μL aliquots of the reaction mixture were removed into 450 μL of an ice-cold solution containing 10 mmol/L EDTA, 10 mmol/L TES, 150 mmol/L NaCl, and 1% (wt/vol) albumin, pH 7.5. Thrombin generated was assayed in a medium containing 10 mmol/L TES, 150 mmol/L NaCl, 0.1% (wt/vol) albumin, pH 7.5, and 100 μmol/L Spectrozyme TH as substrate. From the rate of change in absorbance at 405 nm thrombin activity was calculated using purified thrombin as a standard.

Clotting assays. Gel-filtered C5b67 platelets (and matched-pair controls) were prepared as described above and each suspended to 4 × 10^8/mL in Medium II. In the first stage of the assay, 0.1 mL of platelets (4 × 10^7 cells) were mixed with 0.1 mL of platelet-poor plasma (PPP), C8-deficient PPP (C8D-PPP), or C8D-PPP reconstituted with various amounts (0 to 10 μg) of C8. After incubation for three minutes at 37 °C (to allow C8/C9 binding) clotting was initiated by addition of 0.1 mL each factor Xa (4 × 10^{-10} mol/L) and $CaCl_2$ (25 mmol/L) stock solutions at 37 °C (final volume, 0.4 mL).

Lactate dehyrogenase release Platelet lysis was estimated from the release of cytoplasmic lactate dehydrogenase (LDH) determined under conditions identical to those used in the assay for platelet prothrombinase activity or platelet factor 4 release (below). Enzyme assay was performed according to Wroblewski and LaDue,[17] with total LDH obtained by lysing platelets in 0.1% Triton X-100 (Calbiochem, La Jolla, Calif)

Platelet factor 4 release. Platelet α-granule release was quantitated by platelet factor 4 (PF4) radioimmunoassay (Abbott Laboratories, North Chicago, Ill). 4 × 10^7 gel-filtered platelets (controls or C5b67-treated) were suspended at 37 °C in 0.5 mL Medium III in the presence of either 120 ng C8 + 1.6 μg C9, 5 U thrombin, or 2.5 μmol/L A23187. After ten minutes, 25 μL thrombotect reagent (Abbott) was added, the samples transferred to an ice-water bath (30 minutes), and centrifuged (2500 g) for 20 minutes. The supernatants were saved and immediately assayed for PF4 and LDH

Table 1. Effect of Terminal Complement Proteins on Plasma Clot Formation

	Plasma Clotting Time (sec)*		
Platelets	PPP	C8D-PPP	C8D-PPP + C8†
Control	84.2 ± 1.0	82.0 ± 2.6	82.0 ± 2.0
C5b67	35.3 ± 1.3	84.5 ± 2.3	31.3 ± 1.2
Control + A23187	38.3 ± 1.0	35.1 ± 1.3	N.D

PPP, platelet-poor plasma; C8D-PPP, C8-deficient PPP.

*Mean ± SD (n = 3)

†10 μg C8 added to 100 μL of C8D-PPP.

RESULTS

Effect of complement proteins C5b-9 on platelet procoagulant activity. It has recently been demonstrated that incubation of platelet-rich plasma with zymosan leads to membrane deposition of C3b and to enhanced platelet prothrombinase suggesting a role for serum complement in the modulation of platelet procoagulant activity.[3] In this study, we examined the specific role of the terminal C5b-9 proteins in the procoagulant activity of platelets observed after complement activation. As shown by the data of Table 1, incubation of platelets under conditions leading to membrane assembly of the C5b-9 proteins significantly increased the rate of plasma clot formation. The enhanced procoagulant activity observed for C5b67 platelets suspended in autologous plasma (providing a source of C8 and C9 for membrane assembly of C5b-9) was completely reversed in C8-depleted plasma (Table 1 and Fig 1), confirming that accelerated plasma clotting required binding of the terminal complement proteins (at least through C8) to the platelet surface, and was not due to effects of the added C5b67 proteins per se. As shown by the data of Fig 1, the procoagulant activity observed for C5b67 platelets was restored in a dose-dependent fashion by addition of C8 to C8D-PPP, plasma clotting times approaching that observed for these cells in normal plasma when C8 was added to C8D-PPP in amounts equivalent to the normal plasma concentration of the protein (55 μg/mL). By comparison, clotting times observed for control platelets suspended under these conditions were unaffected by changes in the plasma concentration of C8 (Table 1).

Effect of C5b-9 on platelet prothrombinase. The capacity of the C5b-9 proteins to stimulate assembly of the platelet prothrombinase was investigated in a plasma-free system. C5b67 platelets (or controls) were incubated at 37 °C in the presence of factors Va and Xa, prothrombin, and excess C9, and at the time indicated C8 was added to initiate the assembly of the C5b-9 complex. As illustrated by the data of Fig 2, addition of C8 to the C5b67 platelets (suspended with excess C9) resulted in a rapid increase in the rate of thrombin production. In addition to increasing the platelet's capacity to assemble the prothrombinase, treatment with the C5b-9 proteins (in the absence of Xa, Va, or prothrombin) also resulted in a partial release of platelet factor 4 without causing cell lysis (Table 2), suggesting that prothrombinase activity observed after membrane binding of these proteins may be related to a triggered secretion of α-granule contents. By contrast, prothrombinase activity and PF4 release of control cells was unaffected by C8 and C9 additions. In some experiments, prothrombinase activity of C5b67 cells suspended in the absence of C8 was slightly increased compared to complement-free controls, suggesting either (*a*) a small effect of membrane-bound C5b67 on the rate of prothrombinase or (*b*) the presence of trace amounts of contaminating C8 associated with the gel-filtered platelets.

In Fig 3, the effect of the preformed C5b-9 complex on prothrombinase was measured both in the presence and absence of added factor Va. When no exogenous Va was

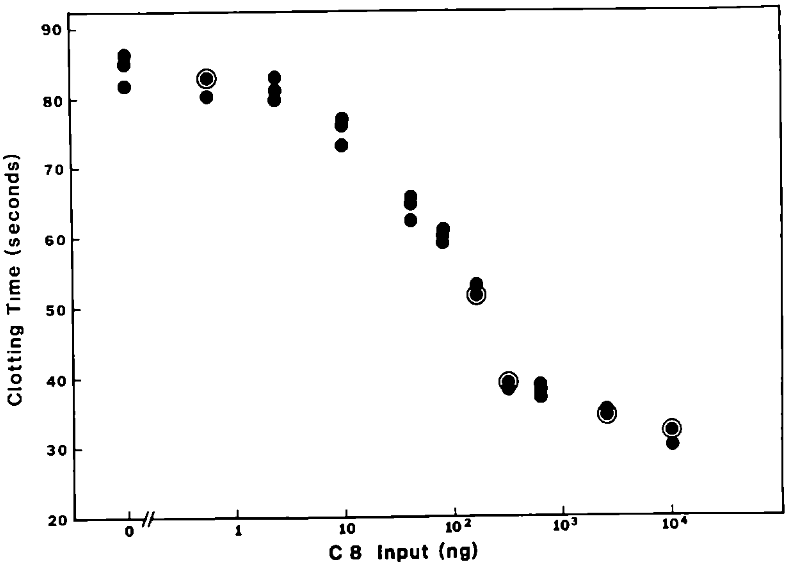

Fig 1. Effect of complement proteins C5b-9 on platelet procoagulant activity. Gel-filtered C5b67-platelets were prepared as described in Materials and Methods and suspended to 4 × 10^8/mL in Medium II. After incubation in C8D-PPP reconstituted with various amounts (0 to 10 μg) of C8, plasma clotting times at 37 °C were measured as described in Materials and Methods.

present (Fig 3A), control and C5b67 platelets produced very small amounts of thrombin, with a lag period of several minutes. Assembly of C5b-9 on platelets augmented prothrombinase activity in a dose-dependent fashion (compare Fig 1) and the lag period before thrombin formation was shortened with increasing input of C8. Following this initial lag phase, maximal rates of prothrombin conversion approaching tenfold control levels were observed at the maximal input of C8 (120 ng per 4 × 10^7 C5b67 platelets) employed in these experiments. Supplementation with factor Va (Fig 3B) resulted in near-linear rates of prothrombin conversion (observed for both control and C5b-9–treated cells) detected immediately after factor Xa additions (ie, no lag phase was apparent). Under these conditions, maximal rates of prothrombin conversion approaching tenfold that of controls were again observed for the C5b-9–pretreated platelets. Under conditions depicted in Fig 3, no cell lysis was detected, even at the highest input of C8 (120 ng per 4 × 10^7 platelets). Incubation of C5b67 platelets with greater amounts of C8 (above 120 ng per 4 × 10^7 cells; not shown) increased the rate of thrombin generation above that shown in Fig 3, but also resulted in a detectable increase in cell lysis above that observed for controls.

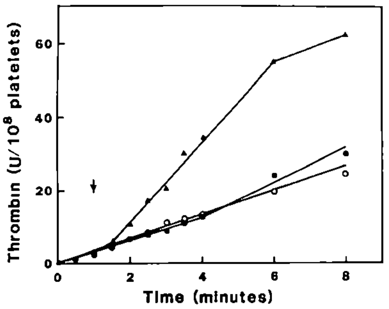

Fig 2. Effect of C5b-9 proteins on platelet prothrombinase activity. Gel-filtered control (○) and C5b67 (●, ▲) platelets were suspended at 37 °C in Medium III at 8 × 10^7/mL in the presence of 2.7 μmol/L prothrombin, 2 nmol/L factor Va and 3.2 μg/mL C9. The reaction was started by addition of 1 nmol/L factor Xa. At time indicated by arrow, C8 (120 ng) was added to C5b67 (▲) and control (○) platelets. At times indicated, 50-μl aliquots were removed and assayed for thrombin as described in Materials and Methods.

Comparison of C5b-9 with other platelet stimuli. The influence of the C5b-9 proteins on the platelet prothrombinase observed under conditions described for Fig 3 was compared to the effect mediated by thrombin and the Ca^{2+} ionophore A23187, two potent platelet activators (Fig 4). Platelets were preincubated (ten minutes, 37 °C) with 1 U/mL thrombin, or 1 μmol/L A23187 to stimulate platelet activation and release reactions,[18] and then tested for their capacity to assemble the prothrombinase. In the presence of exogenous factor Va (B), incubation with 1 U/mL bovine or human (not shown) thrombin elevated prothrombinase activity slightly over that of control platelets, whereas treatment of cells with nonlytic amounts of the C5b-9 proteins resulted in a fivefold increase in the rate of thrombin formation, which was comparable to the response observed after exposure of the platelets to 1 μmol/L A23187 under these conditions. It should be noted that increased rates of prothrombin conversion were observed when the concentrations of C8, thrombin, or A23187 were increased above levels employed in experiments described for Fig 4. Because higher concentrations of these agents also resulted in increased cell lysis (above levels detected for untreated controls), only data for cells treated with 1 U/mL thrombin and 1 μmol/L

Table 2. Platelet Factor 4 Release

Platelets	PF4 Release (ng/10^8 cells)	Lysis (%)
Control	15 ± 6*	0.40
Control + C8/C9	17 ± 5	0.51
C5b67	23 ± 4	0 32
C5b-9	167 ± 35	0.42
Thrombin	1400 ± 72	0.60
A23187	1300 ± 103	1.70
Thrombin + C5b-9	1225 ± 176	1.10

*Mean ± SD

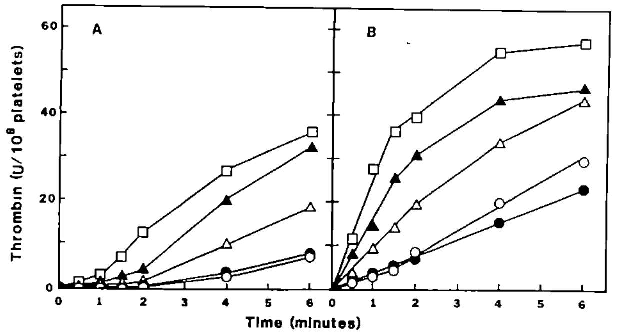

Fig 3. Change in platelet prothrombinase due to C5b-9 assembly: Titration of C8. Gel-filtered C5b67 platelets (4 × 10^7) were suspended in 0.4 mL of Medium III and incubated for ten minutes at 37 °C in the presence of 1.6 μg of C9 and 0 ng (○), 30 ng (△), 60 ng (▲) or 120 ng (□) of C8. After addition of prothrombin (2.7 μmol/L) and factor Xa (1 nmol/L), prothrombinase was measured in the absence (A) or presence (B) of 2 nmol/L factor Va as described in Materials and Methods. Control platelets (●) are also shown. Release of LDH amounted to 1.2% (●), 1.2% (○), 1.3% (△), 1.4% (▲), and 1.2% (□), respectively.

A23187 are shown. By comparison, thrombin generation by platelets incubated with sublytic concentrations of another channel-forming agent, the Na^+/K^+ ionophore nystatin, was indistinguishable from controls. In the absence of added factor Va (Fig 4A), pretreatment of platelets with 1 U/mL thrombin abolished the lag period before onset of prothrombinase activity. By contrast, treatment of the cells with either A23187 or C5b-9 shortened—but did not completely abolish—this lag period. Although the length of the lag phase before prothrombin conversion which was observed in the absence of added factor Va was found to vary from experiment to experiment, in multiple separate experiments performed under conditions described for Fig 4B a distinct lag phase was always observed for those platelets (C5b-9, ionophore-treated, or controls) not previously incubated with thrombin.

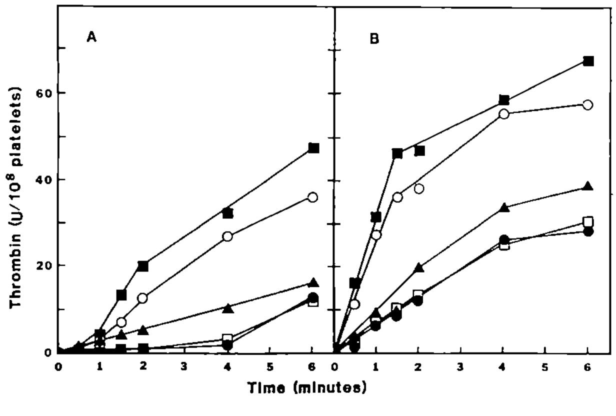

Fig 4. Effect of various platelet stimuli on platelet prothrombinase. Gel-filtered platelets (4 × 10^7) were suspended in 0.4 mL of Medium III and incubated for ten minutes at 37 °C in the presence of 1 U/ml of bovine thrombin (▲), 5 μmol/L nystatin (●) or 1 μmol/L A23187 (■), and C5b67 platelets were incubated with 1.6 μg of C9 and 120 ng of C8 (○). After addition of prothrombin (2.7 μmol/L) and factor Xa (1 nmol/L), prothrombinase was measured in the absence (A) or presence (B) of 2 nmol/L factor Va as described in Materials and Methods. Untreated control platelets (□) are also shown. Release of LDH amounted to 1.2% (□), 1.0% (●), 0.8% (▲), 1.1% (○) and 3.0% (■), respectively.

As illustrated by the data in Fig 5A, treatment of the C5b-9 cells with thrombin abolished the lag period before prothrombin conversion that was observed when these cells were assayed in the absence of exogenous Va. The effect of the individual stimuli with the same platelet suspension are also shown for comparison. Since thrombin also abolished the lag period for control platelets, it is apparent that thrombin pretreatment alone is sufficient to linearize the rate of prothrombin conversion. In Fig 5B, results are shown for the prothrombinase activity of the same platelets when measured in the presence of factor Va. As shown by the data of this figure, a relatively small increase in the rate of thrombin formation was observed when platelets were pretreated with thrombin in addition to the C5b-9 proteins. In three experiments performed under the conditions described for Fig 5B, thrombin was generated at an initial rate of 30.7 ± 1.7 units of thrombin/10^8 platelets × min (mean ± SD) for C5b-9–treated platelets as compared to 49.4 ± 4.1 for platelets treated with both agonists.

DISCUSSION

The data of the present study demonstrate that membrane assembly of the C5b-9 proteins can markedly accelerate platelet-catalyzed thrombin generation, suggesting one mechanism by which the thrombotic reactivity of the platelet can be altered following complement activation. In this context it is of interest to note that (*a*) zymosan treatment of platelet-rich plasma has been shown to induce nonlytic platelet release reactions[2] and to enhance the rate of thrombin formation via the platelet prothrombinase,[3] suggesting that activated component(s) of the complement system alter the platelet procoagulant activity; (*b*) platelet aggregation and release reactions in response to either zymosan or low dose thrombin have been reported to be impaired in plasma deficient in C3, C5, C6, or C7,[19] suggesting that the terminal complement proteins may specifically play a role in platelet

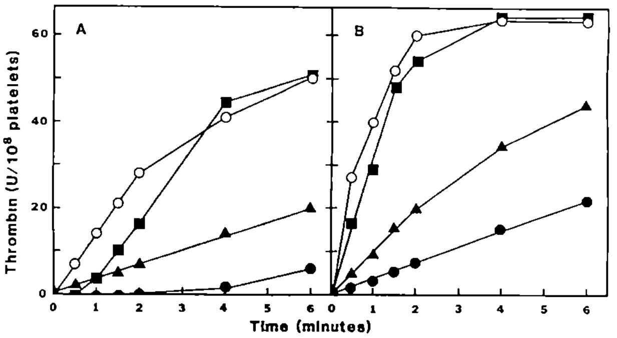

Fig 5. Additive effect of the C5b-9 proteins and thrombin on platelet prothrombinase activity. Gel-filtered C5b67 platelets (4 × 10^7) were suspended in 0.4 mL of Medium III and incubated for ten minutes at 37 °C with 1.6 μg of C9 and 120 ng of C8 in the presence (○) or absence (■) of bovine thrombin (1 U/mL), and control platelets were treated with 1 U/mL of thrombin (▲). After addition of prothrombin (2.7 μmol/L) and factor Xa (1 nmol/L), prothrombinase was measured in the absence (A) or presence (B) of 2 nmol/L factor Va as described in Materials and Methods. Untreated control platelets (●) are also shown.

activation; (*c*) in a plasma-free system, the terminal complement proteins have been shown to potentiate thrombin-stimulated platelet activation[4,5]; and (*d*) there is evidence that the C5b-9 complex spontaneously assembles on the surface of this cell during normal blood clotting.[20] Furthermore, assembly of the purified C5b-9 proteins on gel-filtered platelets has been reported to elevate the intracellular pool of free arachidonic acid, with increased conversion of the fatty acid through the cyclooxygenase pathway.[21] Taken together with the results of the present study, these observations suggest that C5b-9 binding to the platelet surface increases the potential for platelet activation and clot formation.

The capacity of the C5b-9 proteins to accelerate prothrombin conversion in the presence of saturating quantities of factor Va (Figs 2, 3B, 4B, and 5B) closely parallels the effects mediated by the calcium ionophore A23187. As discussed by Zwaal et al,[22,23] elevated prothrombinase activity in cells treated with A23187 can be correlated with an increase in the amount of negatively charged phospholipids available for Va binding to the cell surface, due to transbilayer migration of phosphatidylserine from the cytoplasmic leaflet of the plasma membrane. Elevated prothrombinase activity is also observed after exposure of the cytoplasmic membrane surface due to cell lysis.[22] Under the conditions of our experiments, enhanced prothrombinase activity was observed in response to sublytic quantities of the C5b-9 proteins (release of cytoplasmic LDH unchanged from control levels even at maximal C8 inputs; Fig 4), suggesting that the C5b-9 proteins mimic the effect of calcium ionophores, resulting in a change in the transmembrane distribution of phosphatidylserine In this context it is of interest to note that the C5b-9 proteins have been shown to increase the Ca^{2+}-permeability of the plasma membrane[8,24,25] and to induce plasma membrane vesiculation.[9] In addition to their effect on plasma membrane permeability, the possibility that the proteins directly promote transbilayer migration of phospholipids can also be suggested, based on the capacity of these proteins to disorder the normal lamellar configuration of lipid acyl chains.[26]

The capacity of the C5b-9 proteins to stimulate platelet prothrombinase even in the absence of exogenous Va suggests that C5b-9 also promotes release of platelet factor V, ultimately providing catalytic amounts of the activated cofactor (Va) required for Xa binding to the platelet surface. Triggered release of factor V from the platelet's α-granules has previously been demonstrated following platelet stimulation by arachidonic acid and thrombin, as well as in response to calcium ionophores.[18,27,28] The pronounced lag preceding thrombin generation by C5b-9–treated platelets suspended under these conditions (ie, in the absence of factor Va—Figs 3A, 4A, and 5A) suggests that factor V released from C5b-9–treated platelets is only slowly converted to Va, presumably requiring feedback of either Xa or thrombin on factor V Because the C5b-9 proteins can apparently increase the number of membrane sites for assembly of the prothrombinase as well as promote release of platelet factor V, the net effect of their binding to the platelet surface is to markedly potentiate the effects of thrombin on the activation of the platelet prothrombinase (Fig 5). One might speculate that the increased procoagulant activity of the platelet following membrane deposition of the C5b-9 proteins may account at least in part for episodic vascular thrombosis associated with complement activation in vivo.

In summary, we have demonstrated that the C5b-9 proteins can increase the capacity of the platelet membrane to catalyze thrombin generation by prothrombinase and that this procoagulant activity associated with the terminal complement proteins can occur without lytic rupture of the plasma membrane. Furthermore, our data suggest that the effects of the C5b-9 proteins on platelet prothrombinase may be related to their capacity to act as a calcium ionophore, promoting both the secretion of platelet factor V and the assembly of the functional XaVa complex.

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REFERENCES

1. Sundsmo JS, Fair DS: Relationships among the complement, kinin, coagulation, and fibrinolytic systems. Springer Semin Immunopathol 6·231, 1983

2. Zucker MB, Grant RA: Aggregation and release reaction induced in human blood platelets by zymosan. J Immunol 112:1219, 1974

3. Ozge-Anwar AH, Freedman JJ, Senyi AF, Cerskus AL, Blajchman MA: Enhanced prothrombin-converting activity and factor Xa binding of platelets activated by the alternative complement pathway. Brit J Haematol 57:221, 1984

4. Polley MJ, Nachman RL: The human complement system in thrombin-mediated platelet function. J Exp Med 147:1713, 1978

5. Polley MJ, Nachman RL: Human complement in thrombin-mediated platelet function Uptake of the C5b-9 complex J Exp Med 150:633, 1979

6. Hansch GM, Gemsa D, Resch K: Induction of prostanoid synthesis in human platelets by the late complement components C5b-9 and channel-forming antibiotic nystatin: Inhibition of the reacylation of liberated arachidonic acid J Immunol 35:1320, 1985

7. Wiedmer T, Sims PJ: Effect of complement proteins C5b-9 on blood platelets. Evidence for reversible depolarization of membrane potential J Biol Chem 260:8014, 1985

8. Sims PJ, Wiedmer T: The thrombotic activity of the C5b-9 proteins of serum complement: Role of Ca^{2++} and E_m. Fed Proc 44 551, 1985 (abstr)

9. Sims PJ, Wiedmer T· Repolarization of the membrane potential of blood platelets after complement damage· Evidence for a Ca^{++}-dependent exocytotic elimination of C5b-9 pores. Blood 68:556, 1986

10. Esmon CT: The subunit structure of thrombin-activated factor V. Isolation of activated factor V, separation of subunits and reconstitution of biological activity. J Biol Chem 254·964, 1979

11. Owen WG, Esmon CT, Jackson CM: Conversion of prothrombin to thrombin· Characterization of the reaction products formed during activation of bovine prothrombin J Biol Chem 249·594, 1974

12. Guinto E: Functional properties of bovine factor Va subunits.

PhD Dissertation, University of Oklahoma Health Sciences Center, Oklahoma City, 1983

13. Sims PJ: Complement pores in erythrocyte membranes. Analysis of C8/C9 binding required for functional membrane damage. Biochim Biophys Acta 732:541, 1983

14. Sims PJ: Complement protein C9 labeled with fluorescein isothiocyanate can be used to monitor C9 polymerization and formation of the cytolytic membrane lesion. Biochemistry 23:3248, 1984

15. Sims PJ, Wiedmer T: The influence of electrochemical gradients of Na^+ and K^+ upon the membrane binding and pore forming activity of the terminal complement proteins J Membr Biol 78:169, 1984

16. Wiedmer T, Sims, PJ. Cyanine dye fluorescence used to measure membrane potential changes due to the assembly of complement proteins C5b-9. J Membr Biol 84:219, 1985

17. Wroblewski F, LaDue JS: Lactic dehydrogenase activity in blood. Proc Soc Exp Biol Med 90.210, 1955

18 Kane WH, Lindhout MJ, Jackson CM, Majerus PW: Factor Va–dependent binding of factor Xa to human platelets. J Biol Chem 255:1170, 1980

19. Breckenridge RT, Rosenfeld SI, Graff KS, Leddy JP: Hereditary C5 deficiency in man. III. Studies of hemostasis and platelet responses to zymosan. J Immunol 118:12, 1977

20. Polley MJ, Nachman RL: Ultrastructural lesions on the surface of platelets associated with either blood coagulation or with antibody-mediated immune injury. J Exp Med 141:1261, 1975

21 Polley MJ, Nachman RL, Weksler BB. Human complement in the arachidonic acid transformation pathway in platelets. J Exp Med 153:257, 1981

22 Bevers EM, Comfurius P, Zwaal RFA: Changes in membrane phospholipid distribution during platelet activation. Biochim Biophys Acta 736:57, 1983

23. Comfurius P, Bevers EM, Zwaal RFA. The involvement of cytoskeleton in the regulation of transbilayer movement of phospholipid in human platelets. Biochim Biophys Acta 815:143, 1985

24. Hallett MB, Campbell AK: Is intracellular Ca^{2+} the trigger for oxygen radical production by polymorphonuclear leukocytes? Cell Calcium 5:1, 1984

25. Campbell AK, Morgan BP· Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. Nature 317:164, 1985

26. Esser AF, Kolb WP, Podack ER, Muller-Eberhard HJ: Molecular reorganization of lipid bilayers by complement: A possible mechanism for membranolysis. Proc Natl Acad Sci USA 76:1410, 1979

27. Chesney CM, Pifer D, Colman RW: Subcellular localization and secretion of factor V from human platelets. Proc Natl Acad Sci USA 78:5180, 1981

28. van Rijn J, Rosing J, van Dieijen G: Activity of human blood platelets in prothrombin and in factor X activation induced by ionophore A23187. Eur J Biochem 133:1, 1983

Functional and Immunologic Protein S Levels Are Decreased During Pregnancy

By Philip C. Comp, Gary R. Thurnau, Joy Welsh, and Charles T. Esmon

Protein S, is a natural anticoagulant protein which serves as a cofactor for activated protein C. During pregnancy and in the postpartum period, functional protein S levels are significantly reduced (38% ± 17.3%, mean ± 1 SD) when compared to nonpregnant females (97% ± 31.6%) ($P < 0.001$). In plasma an equilibrium exists between functionally active free protein S and protein S complexed with C4b-binding protein, which is functionally inactive. As a result of this equilibrium either a decreased level of total protein S antigen or an elevation of C4b-binding protein could lead to reduced protein S activity. C4b-binding protein levels measured by enzyme-linked immunoassay are not significantly different in pregnant women versus nonpregnant controls (103.5% ± 21.2% *v* 100% ± 16.9%). However, during pregnancy and in the postpartum period, total protein S levels are reduced (68% ± 10.7%) compared to nonpregnant controls (100% ± 17.0%). This difference is significant at $P < 0.001$. These data demonstrated that the reduction in protein S activity observed during pregnancy is a result of reduced total protein S antigen.

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THE DISCOVERY of two natural anticoagulant plasma proteins, protein C and protein S, has provided insight into the etiology of venous thromboembolic disease. By inhibiting the clotting cascade at the levels of factors V and VIII,[1,2] the enzymatically active form of protein C, protein C_a, regulates blood clot formation. Protein S serves as a cofactor for the anticoagulant effect of protein C_a.[3] Hereditary deficiencies of protein C[4,5] and protein S[6,7,8] have been described in patients with recurrent thrombotic disease. Thrombosis in these patients is presumably due to an inability to regulate and limit the clot formation.

The development of both functional[7,9] and immunologic[4,8] assays for protein C and protein S has facilitated the evaluation of patients with thromboembolic disease. In normal individuals approximately 40% of protein S is found free in plasma and 60% is complexed to C4b-binding protein (C4b-bp), an inhibitor of the complement system.[10] At least two classes of hereditary protein S deficiency have been observed. The first involves reduction of total protein S antigen.[7] The second is characterized by an abnormal distribution between free and C4b-bp–bound protein S, with normal or mildly reduced total protein S antigen levels, and results in a reduction in free protein S.[11] Since only free protein S is functional as a cofactor for activated protein C, both classes of patients have reduced functional protein S levels. Clinically, family members with both classes of deficiency appear to be at increased risk of thrombosis.

In addition to evaluating patients with a history of thromboembolism, these assays permit investigation of patients with medical conditions associated with thrombotic complications. Among these conditions, pregnancy was selected for investigation because pulmonary embolism remains a major cause of maternal death.[12] Furthermore, significant changes in the levels of many plasma proteins, including many of those involved with coagulation,[13] occur during pregnancy. Since protein S is distributed between both free and bound forms, changes in either total protein S or C4b-binding protein could affect protein S activity.

MATERIALS AND METHODS

Proteins. Plasma was provided by the Oklahoma Blood Institute. Protein C, protein S, and C4b-binding protein were prepared as previously described.[7,9,14] An extinction coefficient of 9.3 for C4b-bp was used.[15]

Polyclonal antibodies. A goat was immunized with protein S, and IgG was prepared from the immune serum as previously described.[11] Affinity-purified goat antibodies directed against protein S were purified as follows. Protein S was bound to Affi-Gel 10 (5 mg protein/mL) (Bio-Rad, Richmond, Calif) according to the manufacturer's directions. The IgG in 0.15 mol/L NaCl in 20 mmol/L Tris buffer, pH 7.4, was then passed over a 0.6 × 4 cm column of the immobilized protein S. The column was then extensively washed with 4 mol/L NaCl in 20 mmol/L Tris, pH 7.4, reequilibrated with 0 15 mol/L NaCl in 20 mmol/L Tris, pH 7.4, and the adsorbed IgG eluted rapidly with 0.2 mol/L glycine buffer, pH 2.5. Column fractions were collected in tubes containing sufficient Tris base to bring the pH to approximately 7 to 8.

Monoclonal antibodies. Two murine monoclonal antibodies were prepared[16] against C4b-binding protein and given the designations BP-45 and BP-49, respectively. A monoclonal antibody (designated HPS-2) was prepared against protein S.

Assays. Functional protein S levels were determined as previously described.[7] Total protein S antigen levels were determined by radioimmunoassay. In a manner similar to previously described radioimmunoassays for protein S[17,18] the assay included a prolonged incubation of diluted plasma to promote dissociation of the protein S/C4b-binding protein complex. The reagents employed in the assay were monoclonal (HPS-2) antihuman protein S bound to Immunobeads (Bio-Rad) and ^{125}I-polyclonal affinity-purified anti–protein S. The polyclonal anti–protein S was iodinated by the iodogen method[19] and had a specific activity of 10 μCi/μg. Iodogen was purchased from Pierce Chemical, Rockford, Ill

The radioimmunoassay for protein S was performed in two steps. Standard pooled plasma (from 20 volunteers who were not pregnant or taking oral anticoagulants) was diluted in a serial fashion from 1·100 to 1·800 in 20 mmol/L Tris buffer, pH 7 4, containing 0.15 mol/L NaCl and 10 mg/mL bovine serum albumin. The buffer contained 0.02% sodium azide and had been passed through a

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0.45-μmol/L Millex-HA filter (Micropore, Bedford, Mass) prior to use. Patients' samples were diluted to 1:200. All diluted plasma samples were incubated at 25 °C for 18 hours to promote dissociation of protein S from C4b-binding protein. The assay was conducted by pipetting 50 μL of the HPS_2-Immunobead suspension (containing 4 mg Immunobeads/mL Tris buffer, pH 7.4, containing 0.15 mmol/L NaCl) into 1.5-mL micro test tubes (Bio-Rad) Next, 50 μL of diluted plasma was added and the tube mixed briefly by vortex agitation The tubes were closed and incubated at 25 °C for two hours. At that time, 50 μL ^{125}I-polyclonal affinity-purified anti–protein S diluted to 14 ng/mL in 20 mmol/L Tris buffer, pH 7.4, containing 0.15 mol/L NaCl and 10 mg/mL bovine serum albumin, was added (15,000 cpm/assay tube). The samples were mixed and incubated for four hours at 25 °C. At that time, 1.3 mL 20-mmol/L Tris buffer, containing 2 mol/L NaCl, was added to each tube. The tubes were capped and mixed by vortex agitation for ten seconds and then the Immunobeads were removed by centrifugation in a Microfuge B (Beckman, Palo Alto, Calif) for two minutes. Unbound antibody was removed by aspiration and the wash steps repeated twice. After the final wash, bound ^{125}I-polyclonal anti–protein S was quantitated in a -counter (Nuclear Enterprises, Edinburgh, Scotland). Protein S content of patient samples was calculated from the standard curve. All samples were run in duplicate Within-day variation was 5% and between-day variation was 8%. Patient protein S levels were expressed as percentages of the normal plasma pool level. To determine if C4b-binding protein levels affected the observed protein S level, citrated normal plasma was supplemented with 200 μg/mL and 400 μg/mL purified C4b-bp and allowed to incubate for 18 hours at 25 °C to permit protein S–C4b-bp complex formation (see crossed immunoelectrophoretic analysis below showing reduction of free protein S following incubation). The samples were then assayed for protein S as described above and were found to contain 100% ± 5% of the expected level of protein S.

C4b-binding protein determinations were conducted by an enzyme-linked immunoadsorbant assay employing two monoclonal antibodies (BP-45 and BP-49) which have distinct epitopic specificities against C4b-bp Their specificity was examined by conjugating the respective antibodies to horseradish peroxidase (Type VI, RZ approximately 3.0, Sigma, St Louis) by periodate oxidation.[20] C4b-bp (1 μg/mL in 0.1 mol/L $NaHCO_3$) was adsorbed to 96-well vinyl assay plates (Costar, Cambridge, Mass). After removal of unbound C4b-binding protein by washing, the ability of unconjugated BP-45 to block the binding of conjugated BP-49 was tested. Preincubation of the C4b-binding protein plates for four hours with BP-45 at 25 μg/mL did not decrease the binding of conjugated BP-49. However, preincubation of the plates with 25 μg/mL BP-49 did block binding of conjugated BP-49. In parallel experiments, BP-49 at 25 μg/mL did not block the binding of conjugated BP-45. This suggested that a sandwich ELISA assay employing these two antibodies would prove suitable for measuring C4b-binding protein. Protein S (20 μg/mL) did not block the binding of either antibody

To perform the C4b-bp ELISA, patient plasma diluted to 1:400 and dilutions of normal pooled plasma (1:100 to 1:1,600) were prepared and incubated at 25 °C for 18 hours as described above. To prepare the assay plates the BP-45 antibody was absorbed to 96-well assay plates, the plates blocked with bovine serum albumin, and after washing with 20 mmol/L imidazole buffered saline with 0.02% Tween-20, 50 μL of diluted plasma was applied and incubated at 25 °C for two hours. After washing, 100 μL of 2.5 μg/mL conjugated BP-49 was applied and incubated for two hours. After three washings, the bound conjugated BP-49 was detected with substrate solution (Kirkegaard and Perry, Gaithersburg, Md) Plates were read in a model MR580 Microelisa autoreader (Dynatech, Alexandria, Va). Patient levels were expressed as a percentage of the normal pool. Within-day variation was 9% and between-day variation was 12%.

Rocket immunoelectrophoresis[21] and crossed immunoelectrophoresis[11,22] were conducted as previously described

Patients All participants gave informed consent to participate in the study Pregnant females ranged from 18 to 34 years of age with a median age of 22 years. None had a history of thromboembolic disease. Age-matched controls were not pregnant, not taking oral contraceptives, and had no history of thromboembolic disease Prepartum blood samples were drawn within 24 hours of delivery and postpartum blood samples were obtained 24 to 36 hours after delivery. Statistical analysis of protein S and C4b-binding protein levels was conducted using the Student's t test.

RESULTS

The functional protein S levels of 21 pregnant women during the second and third trimesters were compared with those of 21 nonpregnant controls (Fig 1). The pregnant women demonstrated a mean functional protein S level of 38% ± 17.3% (mean ± SD) with 20 of the 21 study subjects having levels below 58%. The nonpregnant women had a mean functional protein S level of 97.4 ± 31.6% with a range of 52% to 160%; the difference was significant at the $p < 0.001$ level. Functional protein S levels determined immediately prior to and immediately following delivery were similar to those observed during pregnancy.

At least two mechanisms could account for the reduced functional protein S activity. Total protein S antigen could be low, resulting in reduced levels of free (and functionally active) protein S, or C4b-binding protein levels could be increased, leading to reduced levels of free protein by shifting protein S to the complexed (and inactive) form. As an initial assessment, crossed immunoelectrophoretic analysis of plasma protein S was conducted (Fig 2) to obtain a qualitative estimate of the distribution of protein S. During pregnancy, free protein S levels are reduced, as evidenced by the reduced height of the anodally migrating free protein S peak.

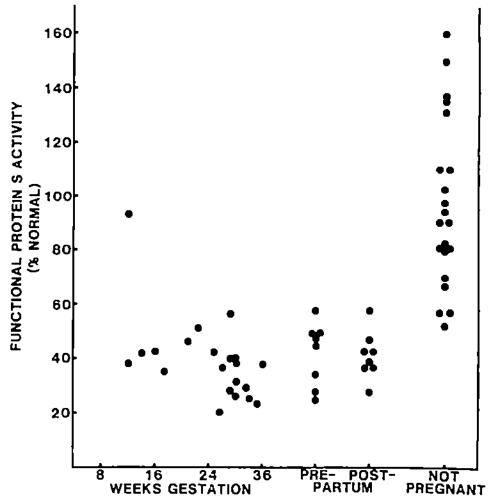

Fig 1. Functional protein S levels during the second and third trimester and pre- and postpartum: The functional protein S level in the pregnant woman was significantly lower ($p < 0.001$) when compared to the controls by the Student's t test.

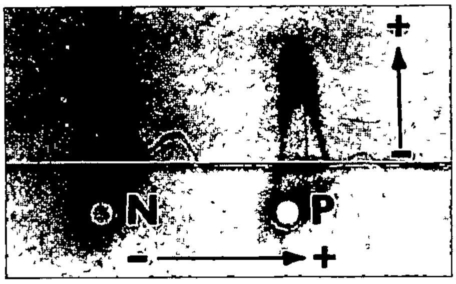

Fig 2. Crossed immunoelectrophoretic analysis of protein S in normal plasma and during pregnancy. On the left, pooled normal plasma (*N*) demonstrates the two immunoprecipitation peaks associated with protein S. The lower, more rapidly migrating peak is formed by the precipitation of free protein S with anti-protein S antibodies. The taller, more slowly migrating peak reflects the protein S associated with C4b-binding protein. On the right is plasma from a pregnant woman (*P*) with a functional protein S activity of 62%. During pregnancy, the peak associated with free protein S is decreased, reflecting reduced levels of free protein in the plasma. In both plasmas the slowly migrating peak demonstrates a broad, diffuse band of immunoprecipitation typical of heterologous dissociating systems. The pattern observed in this pregnant patient's plasma is typical of those seen in the pregnant population. Of the 21 pregnant females examined, 20 had free protein S peaks lower than that observed in pooled normal plasma.

During crossed immunoelectrophoresis the slowly migrating material demonstrates a diffuse band of immunoprecipitation consistent with dissociation of the protein S/C4b-bp complex during the second dimension electrophoresis. To investigate the influence of C4b-bp levels on apparent protein S antigen, plasma was supplemented with C4b-bp (Fig 3). Both crossed immunoelectrophoresis and Laurell rocket immunoelectrophoresis was performed on normal plasma and on the C4b-bp supplemented plasma. Supplementation resulted in almost complete disappearance of free protein S on crossed immunoelectrophoresis. This redistribution also resulted in a 40% decrease in the height of the Laurell rocket. Since the height of the Laurell rockets reflected C4b-bp levels as well as protein S, the method was not utilized to quantitate protein S antigen. Instead, a radioimmunoassay was developed which measured protein S under conditions which favor dissociation of the protein S/C4b-bp complex.[18] This assay required the dilution of plasma to 1:100 with a subsequent 18-hour incubation of the diluted plasma to permit separation of the C4b-binding protein–protein S complex. Under these assay conditions, the C4b-binding protein level does not affect measurement of protein S antigen (see Materials and Methods).

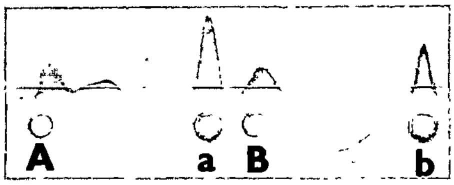

Fig 3. Effect of C4b-BP supplementation on apparent protein S level, as measured by Laurell rocket immunoelectrophoresis. The Laurell rocket pattern of normal plasma is shown above application well "a". The pattern of normal plasma supplemented with 200 μg/mL C4b-BP is shown above well "b". The 40% decrease in the height of the rocket results from complexing of free protein S with C4b-BP. Evidence for increased complex formation is seen in the crossed immunoelectrophoretic patterns of the two plasma samples. Above well "A" (normal plasma) two peaks, reflecting free and complexed protein S are seen; above well "B" (C4b-BP supplemented plasma) only a single peak of complexed protein S is seen. The plasma was incubated with C4b-BP for six hours at 37 °C prior to electrophoresis to permit complex formation. A correction for the dilutional effects of adding C4b-BP was made by adding an identical volume of buffer to the normal plasma sample. Electrophoresis was conducted in the first dimension with plasma in wells "A" and "B". Plasma was placed in wells "a" and "b" immediately before the second dimension electrophoresis.

Employing this assay, the protein S antigen levels in pregnant women averaged 68.4% ± 10.7% of normal (mean ± 1 SD) with a range of 51% to 87%. This was significantly lower ($p < 0.001$) than the protein S levels in nonpregnant controls: 100% ± 16.8% (range 79% to 129%) (Fig 4).

C4b-binding protein levels were not significantly different in the pregnant women (103.5% ± 21.2%) than in the nonpregnant controls (100% ± 16.9%).

Analysis of the functional protein S activity and protein S antigen of the combined pregnant and control population by linear regression analysis demonstrated a positive correlation of 0.56 ($0.05 < p < 0.1$). When protein S functional activity, protein S antigen and C4b-binding protein were analysed by multiple linear regression with the functional activity as the dependent variable, the correlation with protein S antigen was −0.46 and with C4b-binding protein was 0.06.

DISCUSSION

Protein S activity in plasma is dependent on free protein S, not protein S complexed to C4b-binding protein. Evidence for this includes the finding that partially purified protein S/C4b-binding protein complex from normal plasma does not serve as a cofactor for the anticoagulant activity of activated protein C[7] and the finding that the addition of free protein S to protein S deficient plasma restores the activated protein C cofactor activity of the plasma to a greater extent than does the equivalent amount of complexed protein S.[18]

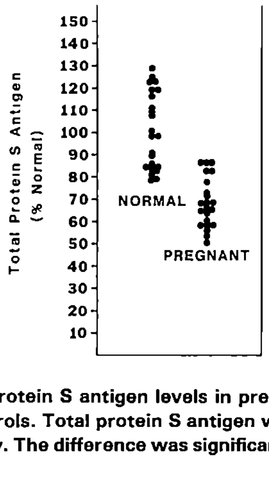

Fig 4. Total protein S antigen levels in pregnant women and nonpregnant controls. Total protein S antigen was determined by radioimmunoassay. The difference was significant at the $p < 0.001$ level.

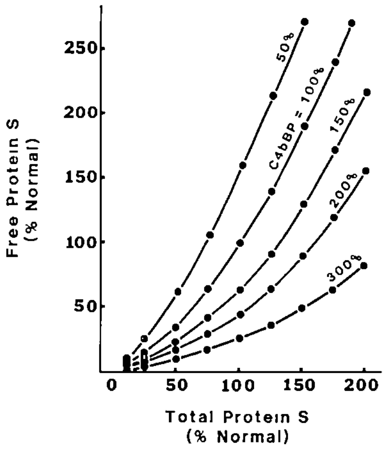

Fig 5. Relationship of free protein S level, total protein S antigen, and C4b-binding protein level. Using the equilibrium relationship K_D = (Free S) (Free C4b-binding protein)/(complex), a quadratic equation may be solved to obtain the predicted free protein S level at given total protein S and C4b-binding protein levels. This was done at five C4b-binding protein levels ranging from 50% to 300% of normal over a range of total protein S levels from 10% to 200% of normal. The protein concentrations and dissociation constant employed were those calculated by Dahlback,[10] ie: C4b-bp 0.28×10^{-6} mol/L, protein S (total) 0.30×10^{-6} mol/L and $K_D = 0.7 \times 10^{-7}$ mol/L. The five resulting curves are labeled with the respective C4b-binding protein level used. As an illustration, at 100% total protein S and 100% C4b-binding protein, the predicted free protein S level is 100% of normal.

The relationship between free protein S level, total protein S antigen, and C4b-binding protein may be calculated from the dissociation constant of the protein S/C4b-binding protein complex, and is shown as a nomogram in Fig 5. Using this nomogram and employing the average total protein S antigen of 68% and the C4b-binding protein levels of 100% calculated for pregnant women, the predicted free protein S level is approximately 50% of normal. This decreased free protein S level is consistent with reduced functional protein S activity observed during pregnancy, which averaged 38%. The dissociation constant used to predict the expected free protein S level was derived using purified proteins and a variety of factors may affect its direct application to human plasma. Furthermore, the plasma protein changes occuring during pregnancy could affect protein S–C4b-BP interaction. This makes obtaining an exact relationship between predicted and observed values in plasma difficult. Nonetheless, this data demonstrates that a reduction in total protein S antigen levels results in a quantitatively larger decrease in functional protein S activity due to redistribution into a complex with C4b-bp.

Why a significant decrease in total protein S occurs during pregnancy is not known. Plasma volume increased by approximately 50% during pregnancy[23] and a dilutional effect may play some role in the reduced protein S levels observed. However, plasma volume increases progressively during pregnancy, plateauing for the last eight weeks before term.[24] Protein S did not show a progressive decrease during pregnancy during gestation (Fig 1), making a simple dilutional effect unlikely. Furthermore, protein C levels were normal in the pregnant women, indicating that dilution of this class-similar vitamin K–dependent protein did not occur. One woman was followed longitudinally at two-week intervals postpartum and the protein S level did not return to normal until 12 weeks after delivery. The dilutional changes associated with pregnancy resolve within the first 2 weeks following delivery. Therefore, it is unlikely that increased plasma volume causes the reduced protein S levels associated with pregnancy.

What the contribution of reduced protein S activity is to the risk of developing thrombophlebitis or pulmonary emboli during pregnancy is unknown. Venous thromboembolic disease does pose a significant risk to women both during pregnancy and in the postpartum period, complicating approximately one in 1,000 pregnancies.[25,26] Changes in the hemostatic system have long been thought to contribute to the risk of this complication. During pregnancy, significant elevations of factors VIII, IX, and X and fibrinogen have been observed.[27,28] Fibrinolytic activity decreases.[29] Changes in these parameters, coupled with significant obstruction of venous outflow from the legs,[30] may contribute to the tendency to develop venous thrombosis during pregnancy. Reduced protein S activity would further shift the hemostatic balance to favor clot formation.

One group of women who may be at a particularly high risk of thrombosis during pregnancy are those who have an inherited protein S deficiency. Pregnancy may worsen the deficiency state and promote thrombosis. Batard et al[31] have identified two women with congenital protein S deficiency whose initial episodes of thrombosis occurred during pregnancy. We have identified three women with similar clinical histories. As more congenitally protein S–deficient families are identified, opportunities to follow protein S levels during pregnancy in congenitally deficient women may become available. At present, determination of protein S levels in women who have developed thromboembolic complications during pregnancy appears reasonable. However, since the levels of protein S activity observed during pregnancy are nearly as low as those in congenitally deficient individuals (15% to 37%),[6] detection of congenital protein S deficiency during pregnancy may prove difficult. Examination of other family members or waiting until well after parturition may be advisable.

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REFERENCES

1. Walker FJ, Sexton PW, Esmon CT: The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. Biochim Biophys Acta 571:333, 1979

2. Fulcher CA, Gardiner JE, Griffin JH, Zimmerman TS. Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. Blood 63:486, 1984

3. Walker FJ: Regulation of activated protein C by a new protein. J Biol Chem 255:5521, 1980

4. Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, Wideman C. Deficiency of protein C in congenital thrombotic disease. J Clin Invest 69:1370, 1981

5. Bertina RM, Broekmans AW, Van der Linden IK, Mertens K: Protein C deficiency in a Dutch family with thrombotic disease. Thromb Haemost 48:1, 1982

6 Comp PC, Esmon CT: Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med 311:1525, 1984

7. Comp PC, Nixon R, Cooper R, Esmon CT· Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest 74.2082, 1984

8. Schwartz H, Fischer M, Hopmeier P, Batard M, Griffin J: Plasma protein S deficiency in familial thrombosis disease Blood 64.1297, 1984

9. Comp PC, Nixon RR, Esmon CT: Determination of functional levels of protein C, an antithrombotic protein, using thrombin-thrombomodulin complex. Blood 63:15, 1984

10. Dahlback B: Purification of human C4b-binding protein and formation of its complex with vitamin K–dependent protein S. Biochem J 209:847, 1983

11. Comp PC, Doray D, Patton D, Esmon CT. An abnormal distribution of protein S occurs in functional protein S deficiency. Blood 67:504, 1986

12. Letsky EA: Thromboembolism in pregnancy and its management Br J Haematol 57:543, 1984

13. Ratnoff OD, Holland TR. Coagulation components in normal and abnormal pregnancies Ann NY Acad Sci 75:626, 1958

14. Nussenzweig V, Melton T: Human C4-binding protein (C4-bp), in Lorand L (ed). Methods in Enzymology, vol 80. Orlando, FL, Academic, 1981, p 124

15. Villiers M-B, Reboul A, Thielens NM, Colomb MG: Purification and characterization of C4-binding protein from human plasma. FEBS Letters 132:49, 1981

16. Galfre G, Milstein C: Preparation of Monoclonal Antibodies· Strategies and Procedures, in Lorand L (ed)· Methods in Enzymology, vol 73. Orlando, FL, Academic, 1981, p 3

17. Fair DS, Revak DJ. Quantitation of human protein S in the plasma of normal and warfarin-treated individuals by radioimmunoassay. Thromb Res 36.527, 1984

18. Bertina RM, van Wijngaarden A, Reinald-Poot J, Poort SR, Bom VJJ: Determination of plasma protein S—the protein cofactor to activated protein C. Thromb Haemost 53:26, 1985

19. Fracker PJ, Speck JC Jr: Protein and cell membrane iodinations with a sparingly soluble chloramine 1,3,4,6-tetrachloro-3a,6adiphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978

20. Wilson MB, Nakane PK: Recent developments in the periodate method of conjugating horseradish peroxidase to antibodies, in Knapp W, Holubar K, Wick G (eds). Immunofluorescence and Related Staining Techniques. Amsterdam, Elsevier/North Holland, 1978, p 215

21. Laurell CB· Quantitative estimation of proteins by electrophoresis in antibody-containing agarose gel Prot Biol Fluids 14:499, 1966

22. Ganrot PO: Crossed immunoelectrophoresis. Scand J Clin Exp Invest (29) 124:39, 1972 (suppl)

23. Rovinsky JJ, Jaffin H: Cardiovascular hemodynamics in pregnancy. 1. Blood and plasma volumes in multiple pregnancy. Am J Obstet Gynecol 93·1, 1965

24. Prichard JA: Changes in plasma volume during pregnancy. Anesthesiology 26:393, 1965

25. Kierkegaard A. Incidence and diagnosis of deep vein thrombosis associated with pregnancy. Acta Obstet Gynecol Scand 62:239, 1983

26. Fell G, Strandress DE· Diagnosis and management of acute venous thrombosis. Clin Obstet Gynecol 24:761, 1981

27 Plass ED, Matthew CW: Plasma protein fractions in normal pregnancy, labor and puerperium. Am J Obstet Gynecol 12:346, 1926

28. Strauss HS, Diamond LK: Elevation of factor VIII during pregnancy in normal persons and in a patient with von Willebrand's disease. N Engl J Med 269:1251, 1963

29 Bonnar J, McNicol G, Douglas A: Fibrinolytic enzyme system and pregnancy. Br Med J iii:387, 1969

30. Ikard RW, Ueland K, Folse R: Lower limb venous dynamics in pregnant women. Surg Gynecol Obstet 132.483, 1971

31. Batard MA, Schwartz HP, Griffin JH· Protein S deficiency associated with thromboembolic disease. Thromb Haemost 54:57, 1985 (abstr)

Trial of Repeated Low-Dose Aspirin in Diabetic Angiopathy

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We compared the ability of aspirin to suppress platelet aggregation and thromboxane synthesis in ten normal subjects and ten patients with diabetic angiopathy and high rate of entry of new platelets into the circulation. When single doses of 100 to 1,000 mg aspirin were ingested daily for 1 month, there were time gaps between doses in which platelets from diabetics and normals aggregated and formed thromboxane ex vivo in response to the combination of arachidonic acid plus collagen. Similar gaps were also found for diabetics, but not for normals, following four daily doses (every six hours) of 25 or 100 mg. Our data show that dose schedules of aspirin which may suffice in normals are not effective in patients with diabetic angiopathy, presumably because these patients have a high rate of entry of new platelets into the circulation. We suggest that continual suppression of platelet thromboxane synthesis and aggregation by low-dose, "slow-release" preparations of aspirin would be an ideal long-term approach for the prevention of thrombosis in patients with a high rate of entry of new platelets into the circulation.

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THERE IS A LARGE body of evidence which suggests that blood platelets are involved in arterial thrombosis and atherosclerosis and that drugs which inhibit platelet function may prevent these disorders.[1] The antithrombotic potential of aspirin is believed to be related to its ability to suppress the synthesis of the cyclic endoperoxides, prostaglandins G_2 and H_2, and thromboxane A_2 (TxA_2), which are potent inducers of platelet aggregation and secretion as well as vasoconstriction.[1-6] However, in spite of extensive experimental and clinical studies, proper dosage and scheduling of this drug remain to be determined.[2-6]

In many clinical trials, aspirin has been administered with widely different doses and schedules.[6-13] Studies in normal subjects[1-4,14-16] have suggested that after the administration of aspirin, the recovery by circulating platelets of the ability to synthesize prostaglandins and thromboxane, as well as the ability to aggregate and secrete, is related to the entry of new platelets into the circulation. Patients with diabetic angiopathy often have an abnormally short platelet survival time and high rate of entry of new platelets into the circulation.[17-24] To inhibit their potential thrombotic role, these new platelets should be continually contacting aspirin. Therefore, we decided to determine whether repeated low doses of aspirin could accomplish this in normal subjects and in patients with diabetic angiopathy.

MATERIALS AND METHODS

Subjects

Because of the complexity of the study, only highly cooperative patients were chosen. Ten diabetic patients (six men, four women, 39 to 51 years old) and ten control subjects matched for sex, age, and weight were studied. These diabetic patients were selected because preliminary studies showed that they had an abnormally high rate of entry of new platelets into the circulation. We determined this by measuring malondialdehyde (MDA) formed by their platelets in response to *N*-ethylmaleimide (NEM) following the method of Catalano et al.[25] An abnormally high entry of new platelets into the circulation determined in this manner correlates with a short platelet survival time determined by radioisotopic methods.[25] Six patients had microangiopathy. Two of them had background retinopathy (ie, hemorrhages, microaneurysms, and hard and soft exudates without any evidence of neovascularization as determined by fluorangiography). two had extensive neovascularization and two had undergone photocoagulation during the previous year. Four patients had macrovascular complications as judged by the presence of S-T wave depression on EKG (four); pathological Q waves (three); absence of peripheral pulses (one), presence of bruits over the carotid vessels (two), or history of angina pectoris or myocardial infarction (three). Patients with macrovascular complications were taking calcium channel blocking drugs. No patient had macro- or microalbuminuria. None of the patients or volunteers smoked. Three patients (1 with macro- and two with microangiopathy) were diagnosed as Type I diabetics because of the occurrence of at least one episode of ketoacidosis and an abnormal C-peptide response to arginine (less than 0.06 pmol/mL C-peptide following intravenous administration of 0.5 g/kg body weight of arginine over a 30-minute period; sensitivity of the method, 0.06 pmol/mL). No difference in the parameters reported in the results section was found between patients with micro- and macrovascular complications or between ketotic and nonketotic subjects. All the patients had been on a 1,500 Kcal diet with a polyunsaturated/saturated fatty acid ratio >1 for at least a year and all were on twice-daily injections of regular plus intermediate monocomponent insulin (46 to 75 U/d, mean 59.2 ± 18.2 SD). None of the controls had a history of disease known to alter platelet aggregation or turnover. Because aspirin is present in so many "over-the-counter" medications, all subjects were asked to avoid cold remedies. In subjects in whom psychoactive drugs were required, seven control and three diabetics, the choice was restricted to Valium. Magnesium and aluminum hydroxides (Maalox) were taken when required for gastric discomfort. Neither patients nor controls had taken any other medication for at least ten days before donating blood. There was no difference between diabetic (188.2 ± 15.7 mg/dL, SEM) and control (196.7 ± 14.9) subjects in serum cholesterol or triglyceride levels (88.7 ± 12.1 mg/dL for patients and 96.9 ± 13.5 for normals). The body mass index for controls was 26.3 and for patients 25.7. Fasting plasma glucose concentrations of patients ranged between 125 mg/dL and 240 mg/dL (mean 187 ± 29). Glycosylated hemoglobin determined by the method of Rahbar[26] ranged between 7.1% and 11.4% (mean 9.0 ± 1.1%) with normal values in our laboratory being 7.1 ± 0.5%. The average duration of diabetes was 8.4 years (range 7 to 13 yrs). Informed consent was obtained from all patients and volunteers after

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approval of the local Human Investigation Committee, and the studies were carried out according to the Principles of the Declaration of Helsinki.

Materials

Adenosine triphosphate (ATP), adenosine diphosphate (ADP) and aspirin (crystalline) were from Sigma Chemical, St Louis. ATP and ADP were dissolved in distilled water and stored in small aliquots at −20 °C. Collagen (col) was from Kollagen reagent Horm, from Hormon-Chemie, München; arachidonic acid (AA, >99% pure) was from Nuchek Prep., Elysian, Minn, and the sodium salt was prepared with 100 mmol/L $NaHCO_3$ The luciferin-luciferase reagent (Chronolume 395) was from Chrono-Log, Havertown, Penn NEM and MDA were from Eastman-Organic Chemicals, Rochester, NY. Highly specific antibodies to TxB_2 (Seragen, Boston) showed less than 0.05% cross-reactivity with prostaglandins E_2, D_2, and 6-Keto-$F_{1\alpha}$. Aspirin used in in vitro aggregation studies was dissolved fresh daily in 0.3 mol/L sodium acetate and kept at 4 °C. For oral ingestion studies of aspirin, Aspirina (500-mg tablets, Bayer Italia, Milano, Italy); Aspirina Pediatrica (100-mg tablets, Bayer Italia) and Vivin C (330-mg tablets of aspirin, Menarini, Firenze, Italy) were used.

Studies on Platelet Aggregation, Secretion, TxB_2 Synthesis and MDA Production

Nine volumes of blood were drawn into one volume of trisodium citrate (3.8%) and platelet-rich plasma (PRP) was prepared by centrifuging blood at 200 g for 15 minutes at room temperature. PRP was then held at room temperature and used within two hours after preparation. Platelet-free plasma (PFP) was obtained by centrifuging fresh PRP in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY) at 12,000 g for five minutes at room temperature. Platelet counts, determined by phase contrast microscopy, were adjusted to 3×10^8/mL by diluting the PRP of both volunteers and patients with PFP. Platelet aggregation and secretion was monitored in a lumi-aggregometer (Chronolog, Havertown, Penn).[14] For each sample and its control, arachidonic acid (1 mmol/L) or an equal volume of vehicle was added in microliter amounts to 0.5 mL of PRP which had been stirring at 1,000 rpm at 37 °C for one minute. Fifteen seconds later, collagen 1 µg/mL or its vehicle was added to the mixture and the extent of aggregation and secretion was determined after three minutes Secretion of ATP was monitored using 50 µl of firefly luciferase and luciferin reagent (10 mg/mL). The sensitivity was such that concentrations of released ATP as low as 0.1 µmol/L could be detected. TxB_2 was measured by radioimmunoassay[15] in aliquots of the supernatant solutions after completion of tests for platelet aggregation and secretion. Sensitivity of the assay was such that as little as 0.5 pmol TxB_2 could be detected. Platelet MDA production after stimulation with NEM (50 mmol/L) was determined in platelets washed and resuspended in protein-free buffer as described by Catalano et al.[25]

Clinical Protocols

Phase 1. Trials of single daily doses of 100, 330 or 1,000 mg aspirin. Blood from all the patients and normal volunteers was collected two days and one day before the initiation of the study. The subjects were then instructed to ingest a 100-mg tablet of aspirin with the evening meal once a day for 1 month, and blood samples were collected twelve to fifteen hours after the first dose and every seven days thereafter. Ingestion of aspirin by all subjects was then stopped and 1 month later a similar trial was initiated with ingestion of 330 mg aspirin daily for 1 month. One month following the end of the 330-mg trial, a similar trial with 1,000 mg daily was carried out

At the completion of each of these studies blood samples were collected every other day for ten days to measure the formation of MDA by platelets.

Phase 2. Trials of multiple doses of 25 or 100 mg aspirin. Preliminary evidence from Phase 1 indicated that single daily doses between 100 mg and 1,000 mg aspirin did not suppress platelet thromboxane synthesis, aggregation, or ATP secretion for 24 hours. Thus, there were long periods between doses during which significant platelet activity was present Therefore we looked for a dosage schedule which would suppress platelet activity continually and two new studies were carried out. In both these studies, after a single loading dose of 100 mg aspirin, 25 mg or 100 mg aspirin were ingested four times daily for 4 weeks. In all subjects blood was collected before the initiation of the study, the day following the first four-times-daily dose schedule, and every week thereafter. Doses were taken at 6 am, 12 noon, 6 pm and 12 midnight. At each visit, blood was collected from each subject at 12 noon and 2 pm (ie, six hours after the morning dose and two hours after the 12 noon dose). As in the Phase 1 studies, at the end of 4 weeks the drug was discontinued and blood was collected every second day for ten days to measure platelet MDA formation.

Statistical Analysis

Student's t test for paired and grouped data was used as appropriate. Nonparametric comparisons showed significant differences similar to those found by Student's t test. Regression analysis was done using a 58C Texas Instrument calculator.

RESULTS

Platelet Aggregation and Secretion After Single Daily Doses of Aspirin (Phase 1)

Twelve to fifteen hours after ingestion of the first dose of aspirin (100, 330, or 1,000 mg), platelets from normal or diabetic subjects did not aggregate or secrete ATP in response to AA (1 mmol/L). However, AA enhanced aggregation and secretion in response to collagen (1 µg/mL) (Fig 1) or to ADP (10 µmol/L) (data not shown). No difference in the amount of the enhancement was found regardless of the dosage of aspirin ingested (Table 1). In platelets from both normals and diabetics preincubation of PRP samples with aspirin (0.5 mmol/L) for 30 minutes at 37 °C abolished the ability of AA to enhance platelet aggregation and secretion in response to collagen. Controls employing similar volumes of the vehicle for aspirin had no effect. Results similar to those obtained after the first dose of aspirin were found at each weekly measurement for each subject.

TxB_2 Formation After Single Doses of Aspirin (Phase 1)

In samples obtained before the administration of aspirin, platelets from diabetics formed 1.5 to 2 times as much thromboxane as those from normals when challenged with AA alone (1 mmol/L) (998 pmol ± 116/3×10^8 platelets in normals, *v* 1738 ± 204 in diabetics, $p < 0.01$). Likewise, there was greater TxB_2 formation ($p < 0.05$) in platelets from diabetics than in those from normals in response to the combination of collagen (1 µg/mL) plus AA (1 mmol/L) (Table 2). Mean concentration of TxB_2 in PFP collected after ingestion of aspirin was below the limit of detection and

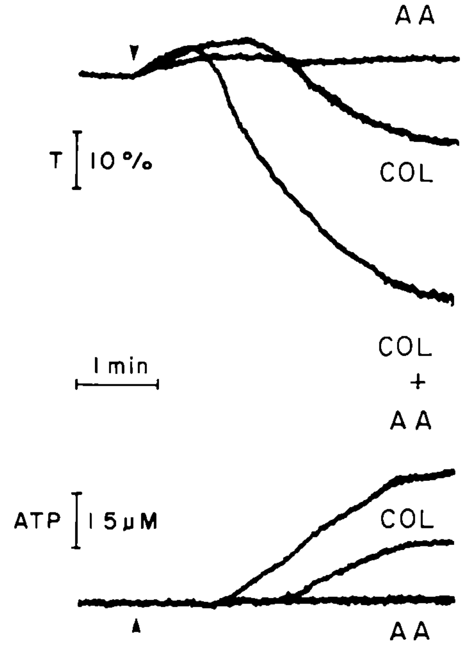

Fig 1. Representative tracings of aggregation and ATP secretion in PRP samples from diabetic patients collected 12 to 15 hours after ingestion of 100 mg of aspirin in single daily doses. In all cases AA was used at 1 mmol/L and collagen at 1 μg/mL. The synergistic effect of AA in combination with collagen is evident in this figure. Aggregation and secretion studies following the administration of single daily doses of 330 mg or 1000 mg of aspirin gave similar results. The arrows indicate the time of addition of the agonist(s). Similar results were obtained in PRP samples from normal subjects.

was unchanged after the addition of collagen (1 μg/mL) plus AA (1 mmol/L). Twelve to fifteen hours after ingestion of aspirin (100, 330, or 1,000 mg), platelets in PRP did not form thromboxane in response to collagen (1 μg/mL), ADP (10 μmol/L) or AA (1 mmol/L) employed alone (data not shown), while TxB_2 was formed in measurable amounts in response to the combination of these agents. No difference in the amount of thromboxane formed in response to collagen plus AA was found when the data obtained after the ingestion of 100 mg were compared to those obtained after 330 mg or 1,000 mg of aspirin (Table 2). Platelets obtained from diabetic subjects after the ingestion of aspirin also formed significantly more thromboxane than platelets from normals. In vitro incubation of PRP (from normals or diabetics) with aspirin (0.5 mmol/L) for 60 minutes at 37 °C abolished thromboxane synthesis, while the vehicle for aspirin did not inhibit thromboxane synthesis.

Aggregation, Secretion and TxB_2 Formation After Repeated Daily Dosing of Aspirin (Phase 2)

Two hours after the noon dose of 25 mg or 100 mg of aspirin (day 2 of the four-times-daily regimen), platelets from normals or diabetics did not form measurable amounts of TxB_2 in response to AA (1 mmol/L), either employed alone or in combination with collagen (1 μg/mL) (Table 3). These platelets did not aggregate or secrete ATP in response to AA (1 mmol/L). In addition, AA did not enhance aggregation and secretion in response to collagen (1 μg/mL) or ADP (10 μmol/L). PRP obtained from normal subjects six hours after the administration of 25 mg or 100 mg aspirin behaved similarly with respect to aggregation and secretion and only formed negligible amounts of TxB_2 (Table 3). In contrast, in PRP samples collected from diabetic patients, TxB_2 was formed in significant amounts (Table 3) and the synergistic effect of AA plus collagen or ADP on platelet aggregation and secretion was observed (data not shown). Incubation in vitro of these PRP samples from diabetics with aspirin (0.5 mmol/L) for 30 minutes at 37 °C suppressed platelet TxB_2 synthesis and the enhancement by AA of aggregation and secretion in response to collagen. Controls (vehicle for aspirin) were without effect. Similar results were obtained at each weekly measurement for each subject.

Return of MDA Formation in Circulating Platelets From Normal and Diabetic Subjects Following Cessation of Aspirin Ingestion

In both patients and normal volunteers, ingestion of aspirin was stopped after 4 weeks and the return of MDA formation by circulating platelets was determined. Fifty percent return of MDA formation in normals was achieved in 4.7 ± 0.2 days (range 3.5 to 5) while in diabetic patients it was achieved in significantly less time; 2.4 ± 0.2 days, $p < 0.05$ (Fig 2). Both in normals and diabetics no differences were found in the recovery of MDA formation with respect to the dosage or scheduling of aspirin and no correlation could

Table 1. Aggregation and ATP Secretion in Response to the Combination of Collagen (1 μg/ml) Plus AA (1 mmol/L) by Platelets in PRP From Normal and Diabetic Subjects 12 to 15 hours After the Ingestion of Single Daily Doses of Aspirin (mean ± SEM)

		After Ingestion of Aspirin (mg)		
Subjects	Before	(100)	(330)	(1,000)
Aggregation (LTU)	6.4 ± 1.3	3.7 ± 0.3*	3.2 ± 0.2*	3.1 ± 0.5*
Normals				
(n = 10)				
ATP secretion (μmol/L)	3.3 ± 0.3	2.1 ± 0.3*	2.0 ± 0.2*	2.1 ± 0.2*
Aggregation (LTU)	6.8 ± 1.2	4.1 ± 0.4*	4.5 ± 0.4*	3.8 ± 0.4*
Diabetics				
(n = 10)				
ATP secretion (uM)	3.6 ± 0.2	2.8 ± 0.2*	2.9 ± 0.2*	2.8 ± 0.3*

n = number of subjects tested.

LTU, light transmission units; 1 unit = 10% light transmittance.

*$p < 0.05$ before v after aspirin (both normals and diabetics). All other comparisons are not statistically significant ($p > 0.05$)

Table 2. TxB_2 Formation in Response to the Combination of Collagen (1 µg/mL) Plus AA (1 mmol/L) by Platelets in PRP From Normal and Diabetic Subjects 12 to 15 Hours after the Ingestion of Single Daily Doses of Aspirin (mean ± SEM)

	TxB_2 Formation (pmol/3 × 10^8 Platelets)			
		After Aspirin Ingestion (mg)		
Subjects	Before	(100)	(330)	(1,000)
Normals (n = 10)	1759 ± 316	36.5 ± 4.3†	27.3 ± 3.7†	31 1 ± 5.3†
Diabetics (n = 10)	2813 ± 209*	58.4 ± 2.5*†	50.1 ± 3.6*†	50 0 ± 6.8*†

n = number of subjects tested

*$p < 0\ 05$ normals v diabetics (both before and after aspirin ingestion)

†$p < 0.01$ before v after aspirin (both normals and diabetics).

The PFP from normals and diabetics contained similar, barely detectable amounts of TxB_2 (0.8 ± 0 1 pmol/mL and 1.0 ± 0.2, respectively). After the ingestion of 100, 330, or 1000 mg aspirin, TxB_2 content was <0.5 pmol/L in all samples of PFP from normals and diabetic patients

be demonstrated between the time at which platelets had recovered 50% of their ability to form MDA and microangiopathy ($r = 0.31$), macroangiopathy ($r = 0.43$) or glycosylated hemoglobin ($r = 0.50$).

DISCUSSION

When used singly at appropriate concentrations in vitro, all naturally occurring aggregating agents induce full platelet aggregation and secretion. Such aggregation and secretion may also be induced by combinations of small amounts of agents at concentrations that are ineffective when used alone.[27] This synergistic effect has been demonstrated in vivo and is likely to play a major role in the early stages of hemostasis and thrombosis. Animals, such as mice, which are very resistant to the thrombotic challenge of intravenous injection of large amounts of single aggregating agents, die with massive occlusion of the lungs by platelet thromboemboli after the injection of very low doses of combinations of aggregating agents.[27]

Prostaglandin endoperoxides and thromboxane must play a critical role in the synergistic effect of combinations of agents in vivo as well as in vitro. For example, extremely low levels of thromboxane in vitro, in combination with other aggregating agents, are sufficient to induce full aggregation and secretion.[14] In addition, aspirin and other cyclooxygenase inhibitors protect mice against the thrombotic effects of intravenous injections of combinations of aggregating agents.[27] Thus aspirin must abolish the synthesis of prostaglandins and thromboxane to achieve a maximal antithrombotic effect. We previously showed that the combination of collagen plus AA is the most potent for revealing the presence of the very small numbers of platelets able to form prostaglandins and thromboxane.[14] Therefore we used this combination to evaluate the effectiveness of single daily doses of aspirin and found that regardless of the dosage of aspirin employed, 12 to 15 hours after the administration of a single dose, when about 98% of thromboxane formation was inhibited, the residual 2% sufficed to enhance aggregation and secretion of platelets from normals (Table 1). Similar results were found in platelets from diabetics (Tables 1 and 2 and Fig 1). Thus, we did not find a single daily dose of aspirin in the range of 100 mg to 1,000 mg able to suppress the synthesis of PG endoperoxides/thromboxane for a full 24 hours. This suggested that the antithrombotic effect of a single large dose of aspirin is limited in time (will not last for

Table 3. TxB_2 Formation in Response to the Combination of Collagen (1 µg/mL) Plus AA (1 mmol/L) in PRP From Normal or Diabetic Subjects Two and Six Hours After the Ingestion of the Last Dose of Aspirin on Day 2 of the Four-Times-Daily Regimen of Aspirin (mean ± SEM)

		TxB_2 Formation (pmol/3 × 10^8 Platelets)	
		Hours After Aspirin Ingestion	
Aspirin Dose (mg)	Subjects	2	6
25	Normal (n = 10)	<0.5	2 1 ± 0.3
	Diabetic (n = 10)	<0.5	39.7 ± 2.2*
100	Normal (n = 10)	<0 5	1.8 ± 0.2
	Diabetic (n = 10)	<0 5	31.1 ± 2 3*

*$p < 0.01$ normals v diabetics

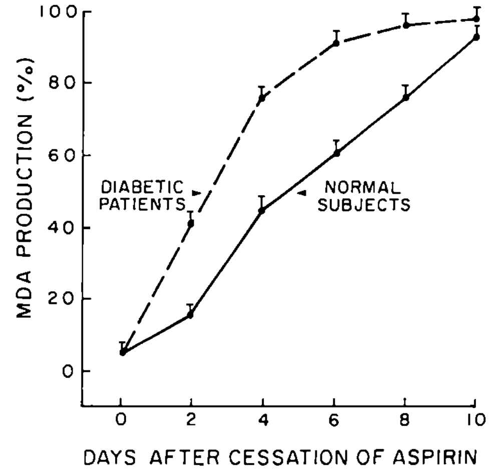

Fig 2. Return of MDA formation in platelets from normal subjects or diabetic patients after cessation of a regimen of a single daily dose of 100 mg aspirin for 1 month. Each point is the mean ± SEM of the data from all the subjects studied. The arrows indicate the time at which circulating platelets had recovered 50% of their original (before aspirin) ability to form MDA.

24 hours) and that repeated small doses of aspirin should be more effective.

It is known that the inhibition of prostaglandin and thromboxane synthesis by aspirin in circulating platelets is an irreversible process.[1–6] Therefore the formation of prostaglandins and thromboxane following the administration of aspirin depends on the entry into the circulation of new platelets whose ability to synthesize prostaglandin endoperoxides/thromboxane has not been affected by contact with aspirin. Studies using platelets labelled with ^{51}Cr indicate that approximately 10% of platelets turn over every day.[25,28] Using combinations of collagen plus AA we saw evidence of newly formed platelets 12 to 15 hours after the administration of aspirin. Within that time period approximately 5% to 6% of newly formed platelets would be in the circulation. Since at least 2% to 3% of platelets that have not contacted aspirin are required to produce concentrations of thromboxane sufficient to act synergistically with other agents,[14] we thought that dosing with aspirin four times a day would suffice to continually abolish the synthesis of thromboxane. In addition to its inhibitory effects on platelets aspirin also inhibits the synthesis by endothelial cells of the vasodilator and antiaggregating agent prostacyclin, and this could limit its usefulness.[1–6] This possible limitation has been countered by observations on the "dose-related" selectivity of aspirin, since single, low, daily doses almost completely suppress TxB_2 synthesis with little apparent effect on prostacyclin formation.[29–33] There has been a recent report on the antithrombotic efficacy of low-dose aspirin.[34] However, the possible role of prostacyclin in thromboresistance is still uncertain.[4,5,35–39] Using a low-dose, four-times-a-day schedule, we found that 25 mg aspirin suppressed thromboxane synthesis and aggregation in response to collagen plus AA by platelets from normals. However, four daily doses of 25 mg or 100 mg still left time intervals in which platelets from diabetic patients aggregated and formed thromboxane in response to the combination. It is unlikely that an impaired sensitivity of circulating platelets to the inhibitory effect of aspirin is involved in this difference since two hours after the administration of 25 mg of aspirin thromboxane synthesis was abolished in platelets from diabetics as it was in normals. In addition, when incubated in vitro with aspirin, platelets from normals and diabetics behaved similarly with respect to inhibition of prostaglandin and thromboxane synthesis. Our MDA data (Fig 2) show that the daily entrance of new platelets into the circulation of diabetic patients is about twice that of normal subjects. It is conceivable that the minimal amounts of cyclic endoperoxides/thromboxane required to potentiate aggregation and secretion in response to collagen are related to the abnormally high rate of entry of new platelets into the circulation of these patients. Newly formed platelets are considered to be larger in size than older platelets[40] and the conversion of AA to prostaglandins and thromboxane is reported to be proportional to the size of platelets.[41] More recently there has been a report indicating that in rabbits young platelets make more thromboxane than older platelets.[42] We found increased synthesis of thromboxane by platelets from patients with an abnormally high rate of entry of new platelets into the circulation. It is also possible that the pharmacokinetics of aspirin may differ in diabetics and normals and so influence our observed findings.

Our observations show that dosage schedules of aspirin based on studies in normal subjects are not applicable to patients with diabetic angiopathy and that doses of aspirin given at relatively long intervals allow for a temporal hiatus in which new platelets entering the circulation cause full aggregation and secretion of ATP in response to combinations of aggregating agents. Thus, continual suppression of platelet prostaglandin and thromboxane synthesis may greatly enhance the antithrombotic potential of aspirin. This has previously been suggested for dosage of aspirin to suppress platelet thromboxane formation without affecting endothelial cell prostacyclin synthesis.[16,17]

Since very frequent oral dosing is not practical, low-dose, slow-release preparations of aspirin which will not allow time gaps in the suppression of prostaglandin endoperoxides and thromboxane synthesis might be the ideal long term approach for the prevention of thrombosis in patients with a high rate of entry of new platelets into the circulation.

In summary, our findings indicate that a small amount of thromboxane can go a long way toward promoting platelet aggregation and a possible thrombotic episode, and that during a course of treatment with high or low dose aspirin there are time periods between doses during which the platelets in PRP from diabetic angiopathic patients can make biologically significant amounts of PG endoperoxides/thromboxane. We suggest that during such time gaps, a thrombotic event could occur. These findings provide the rationale for the continued suppression of cyclooxygenase activity in such patients. This might be accomplished by low-dose, slow-release aspirin.

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REFERENCES

1. Silver MJ: Mechanisms of hemostasis and therapy of thrombosis: New concepts based on the metabolism of arachidonic acid by platelets and endothelial cells. Adv Pharmacol Chemother 18·1, 1981

2. DeGaetano G, Cerletti C, Bertele V: Pharmacology of antiplatelet drugs and clinical trials on thrombosis prevention: A difficult link. Lancet II:974, 1982

3. Salzman EW: Aspirin to prevent arterial thrombosis. N Engl J Med 307:113, 1982

4 Majerus PW: Arachidonate metabolism in vascular disorders. J Clin Invest 72:1521, 1983

5. Marcus AJ: Editorial Retrospective: Aspirin as an antithrombotic medication. N Engl J Med 309.1515, 1983

6. Hirsh J· Progress Review: The relationship between dose of aspirin, side-effect and anti-thrombotic effectiveness. Stroke 61:1, 1985

7. Fields WS, Lemak NA, Frankowski RF, Hardy RJ: Controlled trial of aspirin in cerebral ischemia. Stroke 8:301, 1977

8. Canadian Cooperative Study Group: A randomized trial of aspirin and sulfinpyrazone in threatened stroke. N Engl J Med 299:53, 1978

9 Aspirin Myocardial Infarction Study Research Group: A randomized, controlled trial of aspirin in persons recovered from myocardial infarction. JAMA 243:661, 1980

10. The Persantine Aspirin Reinfarction Study Research Group: Persantine and Aspirin in coronary heart disease Circulation 62:449, 1980

11 Dale J, Myhre E, Storstein O, Stormorken H, and Efskind L: Prevention of arterial thromboembolism with acetylsalicylic acid. A controlled clinical study in patients with aortic ball valves. Am Heart J 94:101, 1977

12. Harter HR, Burch JW, Majerus PW, Stanford N, Delmez JA, Anderson CB, Weertz CA. Prevention of thrombosis in patients on hemodialysis by low-dose aspirin. N Engl J Med 301:577, 1979

13. Lewis HD, Davis JW, Archibald DG, Phil M, Steinke WE, Smitherman TC, Doherty JE III, Schnaper HW, LeWinter MM, Linares E, Pouget JM, Sabharwal SC, Chesker E, DeMots H: Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina N Engl J Med 309:396, 1983

14. DiMinno G, Silver MJ, Murphy S: Monitoring the entry of new platelets into the circulation after ingestion of aspirin Blood 61:1081, 1983

15. Pedersen AK, FitzGerald GA: Dose-related kinetics of aspirin Presystemic acetylation of platelet cyclooxygenase N Engl J Med 311:1206, 1984

16. Reilly IAG, FitzGerald GA: Presystemic inhibition of platelet thromboxane formation by simulated, slow release, low dose aspirin in man. Clin Res 32:320A, 1984 (abstr)

17. Abrahamsen AF: Platelet survival studies in man with special reference to thrombosis and atherosclerosis. Scand J Haematol 3:1, 1968 (Suppl)

18. Ferguson JC, Mackay N, Philip JAD, Sumner DJ: Determination of platelet and fibrinogen half-life with (^{75}Se) selenomethionine: Studies in normal and in diabetic subjects Clin Sci 49:115, 1975

19. Dassin E, Najean Y, Poirier O, Passa P, Bensoussan D: In vivo platelet kinetics in 31 diabetic patients. Correlation with the degree of vascular impairment. Thromb Haemost 40:83, 1978

20. Paton R. Platelet survival in diabetes mellitus using an aspirin-labelling technique. Thromb Res 15:793, 1979

21. Jones RL, Paradise C, Peterson CM. Platelet survival in patients with diabetes mellitus. Diabetes 30:486, 1981

22 Colwell JA, Winocour PD, Lopes-Virella M, Halushka PV: New concepts about the pathogenesis of atherosclerosis in diabetes mellitus. Am J Med 75:67, 1983 (Suppl 5B)

23 Mustard JF, Packham MA: Editorial Retrospective: Platelets and diabetes mellitus. N Engl J Med 311:665, 1984

24. Mustard JF, Packham MA: Platelets and diabetes mellitus. N Engl J Med 297:1345, 1977

25. Catalano PM, Smith JB, Murphy S: Platelet recovery from aspirin inhibition in vivo: Differing patterns under various assay conditions. Blood 57.99, 1981

26. Rahbar S: An abnormal hemoglobin in red cells of diabetics. Clin Chim Acta 22:296, 1968

27. DiMinno G, Silver MJ: Mouse anti-thrombotic assay. A simple method for the evaluation of anti-thrombotic agents in vivo. I. Potentiation of antithrombotic activity by ethyl alcohol. J Pharmacol Exp Ther 225:57, 1983

28. Harker LH, Finch CA: Thrombokinetics in man J Clin Invest 48:963, 1969

29. Patrignani P, Filabozzi P, Patrono C: Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest 69:1366, 1982

30. Preston FE, Whipps S, Jackson CA, French AJ, Wyld PJ, Stoddard CJ: Inhibition of prostacyclin and platelet thromboxane after low-dose aspirin. N Engl J Med 304:76, 1981

31. Hanley SP, Bevan J, Cockbill SR, Heptinstall S: Differential inhibition by low-dose aspirin of human venous prostacyclin synthesis and platelet thromboxane synthesis Lancet 1:869, 1981

32. FitzGerald GA, Oates JA, Hawiger J, Maas RL, Roberts LJ II, Lawson JA, Brash AR. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. J Clin Invest 71:676, 1983

33. Weksler BB, Pett SB, Alonso D, Richter RC, Stelzer P, Subramanian V, Tack-Goldman K, Gay WA Jr. Differential inhibition by aspirin of vascular and platelet prostaglandin synthesis in atherosclerotic patients. N Engl J Med 308:800, 1983

34. Nunez L, Aguado G, Larrea JL, Celemin D, Oliver J: Prevention of thromboembolism using aspirin after mitral valve replacement with porcine bioprosthesis Ann Thorac Surg 37:84, 1984

35. Davis RF, Engelman EG: Incidence of myocardial infarction in patients with rheumatoid arthritis. Arthritis Rheum 17:527, 1974

36. Linos A, Worthington JW, O'Fallon W, Fuster V, Whisnant JP, Kurland LT: Effect of aspirin on prevention of coronary and cerebrovascular disease in patients with rheumatoid arthritis. A long term follow-up study. Mayo Clin Proc 53:581, 1978

37. Curwen KD, Gimbrone MA, Handin RI: In vitro studies on thromboresistance. The role of prostacyclin (PG_2) in platelet adhesion to cultured normal and virally transformed human vascular endothelial cells Lab Invest 42:366, 1980

38. Pareti FI, Mannucci PM, D'Angelo A, Smith JB, Sautebin L, Galli G: Congenital deficiency of thromboxane and prostacyclin. Lancet I:898, 1980

39. FitzGerald GA, Smith B, Pedersen AK, Brash AR: Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N Engl J Med 310:1065, 1984

40. Hirsh J: Laboratory diagnosis of thrombosis, in Colman RW, Hirsh J, Marder VJ and Salzman EW (eds): Hemostasis and Thrombosis. Basic Principles and Clinical Practice. Philadelphia, Lippincott, 1982, p 789

41. Jakubowski JA, Thompson CB, Vaillancourt R, Valeri CR, Deykin D Arachidonic acid metabolism by platelets of differing size. Br J Haematol 53:503, 1983

42. Packham MA, Guccione MA, O'Brien KM: Duration of the effect of aspirin on the synthesis of thromboxane by density subpopulations of rabbit platelets stimulated with thrombin Blood 66:287, 1985

Immunoreactive Erythropoietin Concentrations in Fetal and Neonatal Rats and the Effects of Hypoxia

By Gisela K. Clemons, Sherry L. Fitzsimmons, and Darlene DeManincor

Immunoreactive erythropoietin (Ep) was measured in normoxic and hypoxic (0.5 atm; 18 hours) fetal rats from day 14 to day 21 of gestation and in neonatal rats from birth to weaning, and was compared to the adult rat. Amniotic fluid (AF) Ep was approximately 100 mU/mL on day 14 and 15, and decreased to 20 mU/mL on day 20, with no difference between the hypoxic and normoxic mothers. Only on day 21 did the Ep in the AF increase slightly in the hypoxic group, while the Ep in the control group continued to fall to 15 mU/mL on day 21, the last day of pregnancy. Before day 17 of gestation the rat fetus appears to have hypoxia-independent, extrahepatic Ep available which is followed by hepatic and renal Ep production, both of which become sensitive to maternal hypoxia during the last days of pregnancy. In the neonatal rat plasma and tissue, Ep levels varied greatly during the first three weeks of life regardless of whether the animals were hypoxic or not. With the exception of the first and ninth days of life, circulating Ep levels were higher than adult levels in normal newborn rats. Neonatal rats responded to hypoxia with increasing Ep levels, and the response increased with age such that during the third week of life the plasma Ep levels were significantly higher than in adult hypoxic rats. No sex difference in male and female response to hypoxia could be documented until sexual maturity (day 42). In the normoxic neonatal rat more Ep originated from the liver than the kidneys until day 10, while under hypoxic conditions the switch occurred as early as two days after birth.

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PREVIOUS STUDIES have established that erythropoiesis in the fetal and adult mammal is regulated by erythropoietin (Ep).[1-3] It has also been shown that fetal Ep production responds and increases during periods of fetal hypoxemia. Zanjani et al[4] have provided evidence that the liver is the primary site of Ep production in the fetus, at least in the sheep during the last trimester, and the switch from hepatic to renal Ep synthesis occurs around the time of birth,[5] whereas in the rat previous studies showed that Ep is predominantly derived from the liver until after birth.[6-8] While it has been shown that the neonatal rat is able to increase Ep levels in response to hypoxic hypoxia,[9-12] erythropoiesis cannot be further stimulated in the newborn rat by exposure to hypoxia or exogenous Ep administration,[13-16] nor can erythropoiesis be suppressed in these young animals by starvation, bilateral nephrectomy, or hypertransfusion.[6,9,17-20] Several investigations have suggested that erythropoiesis in the neonatal rat may be independent of renal Ep during the first three weeks of life. These studies were carried out in several different laboratories, using animals at different ages and atmospheric pressures for varying lengths of time and the samples were analyzed mostly in the in vivo polycythemic mouse assay.

The high sensitivity of the radioimmunoassay and the relatively small sample size required for analysis made these studies possible. We attempted to identify the tissue(s) responsible for Ep production in the normal rat fetus and to determine the effects of maternal hypoxia on Ep production during the last week of fetal life. In addition we tried to answer the following questions: what are the circulating, hepatic, and renal Ep levels in normal neonates measured daily through the first three weeks of life and when they attain sexual maturity (day 42)? How are these Ep levels altered by exposure to hypoxic hypoxia? Can immunoreactive Ep be measured in milk obtained from hypoxic lactating rats, and can it be transferred to the neonatal animals? We also attempted to determine the age at which the switch from the liver to the kidney occurred both in the normal and hypoxic neonatal rat.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats with timed pregnancies, obtained from Simonson Laboratory, Gilroy, Calif, were housed with free access to food and water for at least five days before the start of the experiments (day 8 to day 9 after mating). For the neonatal study the cages were checked two days before the presumed delivery date every two hours from 7 am to 8 pm, and the time and litter size was recorded. Approximately 75% of the deliveries occurred between 7 am and 2 pm. The litter size ranged from four to 15 pups and was adjusted to eight to ten per mother in order to assure equal nutritional status for the neonates during the experiment. When pups reached the age of 10 days, mothers were selected for hypoxia each day such that the same number of male and female pups could be exposed. All animals in the hypoxic groups were exposed to a high altitude equivalent to 18,000 feet (0.5 atm) for 18 hours (3 pm to 9 am). Every day hypoxic mothers, fetal rats, and neonatal rats were killed within one hour after hypoxia (9 to 10 am) and immediately followed by control animals for each group (10 to 11 am)

Experimental Design

Fetal study From day 13 of gestation, each day four pregnant rats were exposed to hypoxia. The mothers were bled by cardiac puncture under ether anesthesia and amniotic fluid was aspirated before the fetus was removed from the uterus. Placental tissue was

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not collected because we had established earlier that placental Ep content did not exceed 20 mU/g at any time after 14 days of gestation. The litter size varied from eight to 13 animals. All fetuses were blotted dry and weighed before tissues and blood were obtained Fetal blood, liver tissue, and kidney tissue were collected as early as possible and pooled for each litter. Fetal liver tissue was collected from day 15 on, blood from day 17 and kidney tissue from day 19 of gestation. On day 14 tissue could not be obtained for analysis; therefore Ep was measured in the whole fetus and on days 15 to 18 in fetuses without liver and kidney anlage from which most of the blood had been removed. Plasma from each litter was pooled after centrifugation with the exception of day 17 from the hypoxic group. Plasma pools from all fetuses of four mothers had to be pooled in order to make repeated radioimmunoassay (RIA) analysis possible.

Neonatal study. Mothers and their pups were exposed to hypoxia; each day an age-matched group served as controls. From one to 10 days of age blood was collected in heparinized hematocrit tubes after decapitation. Older animals were bled by heart puncture. From day 1 to day 21 six pools were generated for the control and hypoxic group, except that in the latter group six pools were obtained for males and females separately from day 10 to day 21. Samples had to be pooled to make RIA analysis possible. During the first four days four animals were pooled, three from days 5 to 7, and thereafter until day 21 two animals were sufficient. On day 42 analysis was possible on individual animals and eight animals were used in each group 8 control, 8 hypoxic male, and 8 hypoxic female rats. The kidney and whole liver tissues were excised and pooled identically to the pups whose plasmas had been pooled Salivary glands were collected and combined for all animals in each group: controls, hypoxic (days 1 through 9), and hypoxic males and females (days 10 through 21 and day 42)

During the collection time the tissues were stored on ice in small covered dishes in order to reduce possible degradation and evaporation and homogenized as fast as possible, although this step was slower than obtaining the tissues. All tissues were weighed and homogenized in 0.05 mol/L phosphate buffer (pH 7.5) with 5% fetal bovine serum (wt/vol), and centrifuged at 10,000 g for 15 minutes in an Eppendorf Micro Centrifuge (Sybron-Brinkman Instruments, Palo Alto, Calif). Plasma, amniotic fluid, and tissue homogenates were stored frozen at −20 °C until RIA analysis.

Since the neonatal animals were exposed to hypoxia with their mothers, it had to be ascertained whether the levels measured in the pups could have been derived from the mother via the milk. In order to do this, two groups of experiments were carried out In the first experiment three lactating rats were removed from their litters and exposed to hypoxia overnight for 18 hours. After returning to ambient pressure, they and three control rats were anaesthetized with pentobarbital (60 mg/kg IP) and lactation was induced by injection of pitocin (4 to 5 U/animal), obtained from Sigma Chemical, St Louis. Within five to ten minutes milk could be obtained slowly by mild suction through a glass pipette connected to a vacuum source.

In a second experiment groups of pups at the age of seven days were separated from their mothers. Three adults were exposed to hypoxia overnight while three others were kept in the colony at ambient pressure. At 8 am the pups were returned for nursing for three hours to both groups of mothers. The animals were killed three hours later and plasma, kidneys, and liver tissues were collected and analyzed for Ep content.

Radioimmunoassay of Rat Erythropoietin

Immunoreactive erythropoietin concentrations were measured by RIA [21] Even though the tracer is highly purified Ep of human origin and the antiserum was generated against human urinary Ep extract, a bioassayed standard preparation of high-altitude rat serum yields a dose-response curve in the RIA similar, although not identical, to that obtained with the Second International Reference preparation of human Ep. The technical details of the RIA and the validation of the rat Ep RIA have been published.[22] The sensitivity limit of this assay is approximately 4 mU/mL, interassay variation is <12% and intrasssay variation <9%. All samples were initially analyzed in single 100-μL aliquots and repeated in two separate RIAs in duplicates, sample size permitting, at appropriate dilutions that could be compared to the standard curves. The results presented in Table 1 represent the means of each of the four pools generated each day per group and three to five determinations per pool. The neonatal data in Table 2 are the means of four determinations from the latter two assays. Because the tissues were homogenized in an equal volume of buffer to tissue weight, this volume in the RIA represented a 1·2 dilution and was accounted for in the computer program as such. In addition, some plasma and kidney tissue homogenates were serially diluted as a check for parallelism. Results were analyzed with the sigmoid computer program developed by Rodbard and Hutt[23] on the LBL CDC 7600 Computer.

RESULTS

Erythropoietin Concentrations in Fetal Plasma, Tissues, and Amniotic Fluid

There appears to be no immunological difference between fetal and adult Ep because all sample dilutions parallel the rat Ep standard. The Ep concentrations measured in the whole fetus on day 14 were relatively high and appear to be in equilibrium with the amniotic fluid (AF) (Table 1). Thereafter a steady decline in Ep levels was measured in both fetuses and AF with advancing pregnancy. This is particularly interesting in light of the fact that the volume of the AF diminishes during the last week of gestation and becomes increasingly more viscous. Amniotic fluid of control animals reached the lowest value on day 21, a time at which there was the first significant increase of Ep measured in the hypoxic group due to their increased urinary contribution ($p < 0.05$). There was no difference between the Ep concentrations in the AF as a result of maternal hypoxia with the exception of day 21. Similarly, no significant difference could be measured in whole pups on day 14 of gestation or later when most of the plasma, all of the liver, and later all of the kidney had been removed.

On day 15 maternal exposure to hypoxia did not affect Ep production in fetal liver tissue and the increase measured on day 16 is still statistically insignificant. Fetal hepatic Ep production in response to maternal hypoxia, however, could first be demonstrated in tissue content on day 17 ($p < 0.01$). Throughout the remainder of gestation liver Ep concentrations were significantly higher in the hypoxic fetuses than in those from control mothers. Ep content in control liver tissue remained fairly stable between days 15 and 19. Beginning with day 19 there was a significant decline in hepatic tissue Ep concentration, both in the control and hypoxic groups, and this decline was reflected in the lowest plasma values on day 20.

The earliest obtainable renal tissue on day 19 was also not affected by maternal hypoxia but increased its competence of

Table 1. Erythropoietin Concentrations in Fetal Rats and the Effect of Maternal Exposure to Hypoxia (HA)

Fetal Age (days)	Pups/ Pool*	Plasma (mU/mL)		Liver (mU/g)		Kidney (mU/g)		Pups (mU/g)		Amniotic Fluid (mU/mL)		Maternal Plasma (mU/mL)	
		C	HA	C	HA	C	HA	C	HA	C	HA	C	HA
14	8–11	—	—	—	—	—	—	80.2 ± 6.5	78.4 ± 7.8‡	99.2 ± 3.0	87.4 ± 9.5	20.3 ± 1.6	322 ± 54¶
15	9–12	—	—	14.4 ± 1.5	13.5 ± 0.8	—	—	61.7 ± 2.1	68.2 ± 6.4§	101.0 ± 3.2	109.1 ± 5.5	20.1 ± 1.8	476 ± 71
16	8–12	—	—	13.4 ± 0.9	20.3 ± 3.7	—	—	45.9 ± 1.6	53.8 ± 3.2§	90.8 ± 9.9	92.1 ± 5.7	31.7 ± 3.5	446 ± 59
17	8–13	32.6 ± 4.0†	42.5 (1)	16.5 ± 1.7	30.6 ± 3.3**	—	—	27.9 ± 1.1	37.6 ± 2.1‖	70.9 ± 2.4	84.0 ± 3.4	25.2 ± 5.3	336 ± 13
18	8–11	28.0 ± 2.1	56.1 ± 4.5††	11.8 ± 1.3	37.8 ± 8.3††	—	—	28.1 ± 2.9	19.0 ± 0.7‖	68.3 ± 8.8	52.3 ± 1.8	31.2 ± 6.0	308 ± 50
19	8–12	6.6 ± 1.6	26.6 ± 2.2††	10.2 ± 0.9	17.5 ± 2.7#	6.8 ± 1.3	8.2 ± 0.7	8.5 ± 1.0	15.7 ± 2.3‖	40.5 ± 7.5	48.9 ± 1.1	29.1 ± 7.0	288 ± 45
20	7–11	4.2 ± 0.2	10.2 ± 1.7#	7.9 ± 0.7	15.0 ± 1.8#	7.8 ± 0.1	21.9 ± 4.6#	—	—	20.5 ± 1.6	22.4 ± 1.3	25.6 ± 3.3	495 ± 100
21	8–12	8.6 ± 0.7	38.6 ± 4.8††	7.7 ± 0.4	20.8 ± 1.3††	9.3 ± 0.7	34.7 ± 7.6#	—	—	15.1 ± 5.1	26.6 ± 1.9#	20.8 ± 3.2	197 ± 24

*Each determination consisted of four pools with the exception of fetal HA plasma on day 17.

†Mean ± SEM

‡Whole pup.

§Pup minus liver tissue.

‖Pup minus liver and kidney tissue.

¶All HA values are significantly higher ($p < 0.001$).

The index shown for significance is always between the HA group and control and not within each group #$p < 0.05$; **$p < 0.01$; ††$p < 0.001$.

Table 2. Erythropoietin Concentrations in Neonatal Normoxic and Hypoxic Rats

Age (days)	n	Plasma (mU/mL)		Kidney (mU/g)		Liver (mU/g)	
		Control	Hypoxia	Control	Hypoxia	Control	Hypoxia
1	6(4)	18.8 ± 0.5	89.5 ± 8.9	13.5 ± 1.9	103 ± 5.4	11.1 ± 2.0	38.9 ± 7.2
2	6(4)	44.7 ± 5.3	336 ± 33	10.9 ± 2.4	314 ± 21	54.5 ± 8.1	37.4 ± 9.4
3	6(4)	48.1 ± 6.1	566 ± 66	9.4 ± 1.3	512 ± 65	28.5 ± 6.4	122.0 ± 15
4	6(4)	57.1 ± 7.7	647 ± 73	24.4 ± 4.1	570 ± 82	21.1 ± 5.3	167.0 ± 13
5	6(3)	83.9 ± 8.2	676 ± 82	31.1 ± 4.1	832 ± 67	16.2 ± 3.3	142.0 ± 9.8
6	6(3)	55.2 ± 4.2	949 ± 29	17.6 ± 3.0	964 ± 33	13.8 ± 4.2	243.0 ± 29
7	6(3)	55.6 ± 6.1	1061 ± 56	15.7 ± 2.4	1009 ± 79	11.2 ± 1.6	221.0 ± 10
8	6(2)	44.9 ± 3.2	1164 ± 143	12.7 ± 1.7	1142 ± 137	10.3 ± 0.4	215.0 ± 24
9	6(2)	26.2 ± 3.0	763 ± 60	9.7 ± 0.8	746 ± 100	6.7 ± 0.9	200.0 ± 44
10	6(2)	188.0 ± 24	464 ± 39 M 564 ± 37 F	155.0 ± 35	281 ± 33 M 458 ± 40 F	24.5 ± 3.1	74.5 ± 9.0 M 92.9 ± 14.6 F
11	6(2)	60.8 ± 8.0	1363 ± 167 M 1275 ± 156 F	32.4 ± 6.4	971 ± 76 M 1362 ± 119 F	23.4 ± 7.8	158.0 ± 13 M 215.0 ± 25 F
12	6(2)	45.6 ± 4.3	1273 ± 216 M 1034 ± 214 F	18.4 ± 3.6	1263 ± 204 M 906 ± 225 F	6.7 ± 0.9	287 ± 51 M 148 ± 34 F
13	6(2)	106.0 ± 6.2	860 ± 134 M 942 ± 91 F	65.2 ± 10	393 ± 43 M 552 ± 77 F	10.7 ± 1.2	80.2 ± 11.8 M 92.3 ± 11.5 F
14	6(2)	58.5 ± 4.5	1681 ± 139 M 1389 ± 38 F	27.6 ± 3.8	1249 ± 133 M 831 ± 80 F	8.9 ± 0.7	244.0 ± 40 M 190.0 ± 44 F
15	6(2)	130.0 ± 9.7	1435 ± 113 M 1501 ± 164 F	51.6 ± 5.5	1020 ± 86 M 1253 ± 159 F	13.0 ± 1.6	145.0 ± 14 M 186.0 ± 29 F
16	6(2)	109.0 ± 13	2174 ± 257 M 2213 ± 206 F	62.3 ± 7.6	2148 ± 185 M 2867 ± 265 F	11.1 ± 1.7	249.0 ± 14 M 279.0 ± 41 F
17	6(2)	112.0 ± 17	2087 ± 180 M 1643 ± 168 F	49.0 ± 9.0	2094 ± 63 M 2051 ± 237 F	12.5 ± 1.4	231.0 ± 43 M 206.0 ± 14 F
18	6(2)	58.7 ± 4.8	2244 ± 174 M 1938 ± 63 F	27.2 ± 5.8	1967 ± 152 M 1757 ± 130 F	9.9 ± 1.4	205.0 ± 22 M 162.0 ± 29 F
19	6(2)	190.0 ± 19	1817 ± 85 M 1701 ± 135 F	121.0 ± 14	1906 ± 69 M 2002 ± 207 F	14.4 ± 4.7	149.0 ± 19 M 174.0 ± 33 F
20	6(2)	126.0 ± 12	1882 ± 191 M 2151 ± 136 F	87.7 ± 13	1043 ± 67 M 2266 ± 165 F	12.8 ± 3.8	173.0 ± 18 M 153.0 ± 9.2 F
21	6(2)	127.0 ± 8.8	2008 ± 148 M 1986 ± 67 F	105 ± 10	1457 ± 92 M 2051 ± 231 F	13.2 ± 1.7	161.0 ± 20 M 213.0 ± 28 F
42	8(1)	22.8 ± 1.3	902 ± 79 M 318 ± 28 F	10.8 ± 1.7	434 ± 63 M 186 ± 26 F	5.5 ± 0.7	54.6 ± 6.1 M 18.5 ± 1.8 F

M, male, F, female.

Ep elaboration significantly on day 20 and 21 with a three fold elevation over that measured in control kidneys.

Erythropoietin Concentrations in Normoxic Neonatal Rats

The results of this study are shown both graphically and in tabulated form. Plasma Ep levels in normoxic neonatal rats vary profoundly during the first three weeks of life (Table 2 and Fig 1) With the exception of days 1 and 9 the circulating levels in the neonates were significantly higher than those measured in 42-day-old and adult rats. Peak values of 190 mU/mL were measured on days 10 and 20. Liver tissue Ep concentrations rose fivefold on the second day of life and are most likely responsible for the increase observed in plasma Ep levels. Thereafter the high plasma peaks also coincided with elevated renal Ep concentrations, the most pronounced occurring on day ten. After the second day of life liver Ep levels decline to less than 20 mU/g of tissue with the exception of days 10 and 11, when a small increase could be observed.

Erythropoietin Concentrations in Hypoxic Neonatal Rats

Hypoxic exposure showed that neonatal rats increase Ep production as early as the first day of life, and circulating Ep levels closely resembled renal Ep concentrations (Fig 2). While on the whole Ep production increased with age it is of interest that on days 10 and 13 we observed sharp declines in renal and plasma Ep in hypoxic animals, at a time when unstimulated control animals showed unexpected peaks. Nevertheless, the Ep levels in the hypoxic groups were still significantly higher than those measured in the controls. Liver Ep concentrations fluctuated relatively little. No sex difference in the response of males and females to hypoxia could be documented from day 10 through weaning, but at day 42 the levels in male rats were significantly higher than in female rats and were similar to those measured in adult rats of both sexes. The sharp decline on days 10 and 11 also cannot be attributed to a female response because, as can be seen in Table 2, even though they were all reduced, plasma, kidney, and liver content of the female group was actually higher than that of the male rats. Analysis of salivary gland

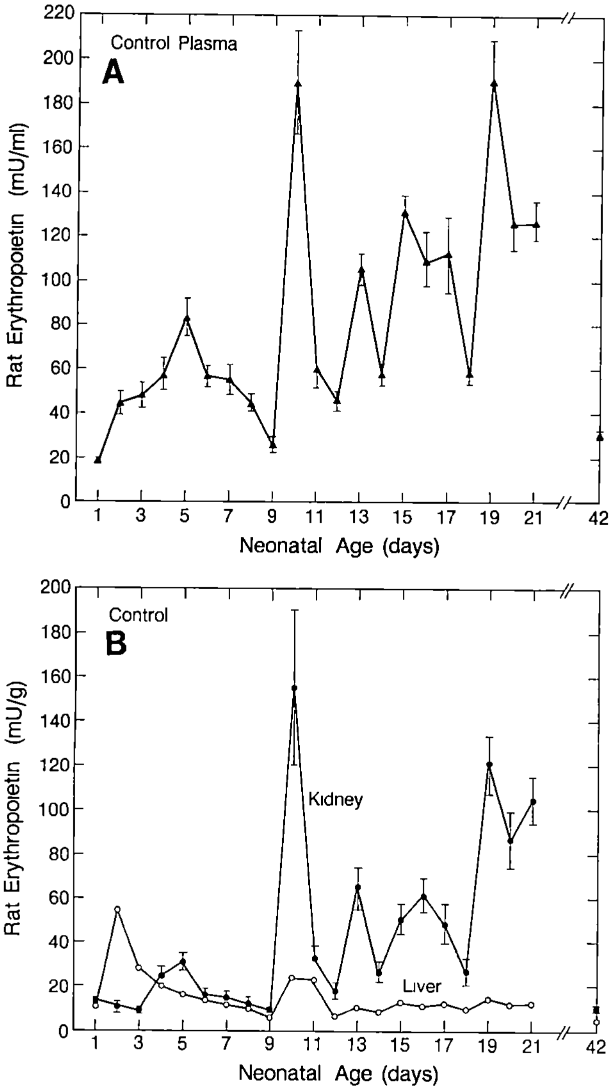

Fig 1. (A) The daily variation in plasma erythropoietin in the normoxic neonatal rat. (B) The liver and kidney erythropoietin concentrations during the same period. Values are means ± SEM.

tissue homogenates for the presence of immunoreactive Ep were negative throughout the study from one to 42 days of age.

Total Renal and Hepatic Erythropoietin Content and Time of Switching

Taking into account tissue weights and their Ep concentrations, it is possible to calculate how much of the hormone is present in renal and hepatic tissues. Figure 3 shows that in the normoxic neonatal rat the Ep content in the liver is higher than in the kidney until day 10. The profound increase on day 10 seems to be connected with the liver-to-kidney switch of Ep production in the rat, because thereafter renal Ep content is always higher than liver content. During hypoxic conditions, however, the switch occurred as early as two days after birth, and in spite of increased liver weight from day 2 on, kidney content was higher.

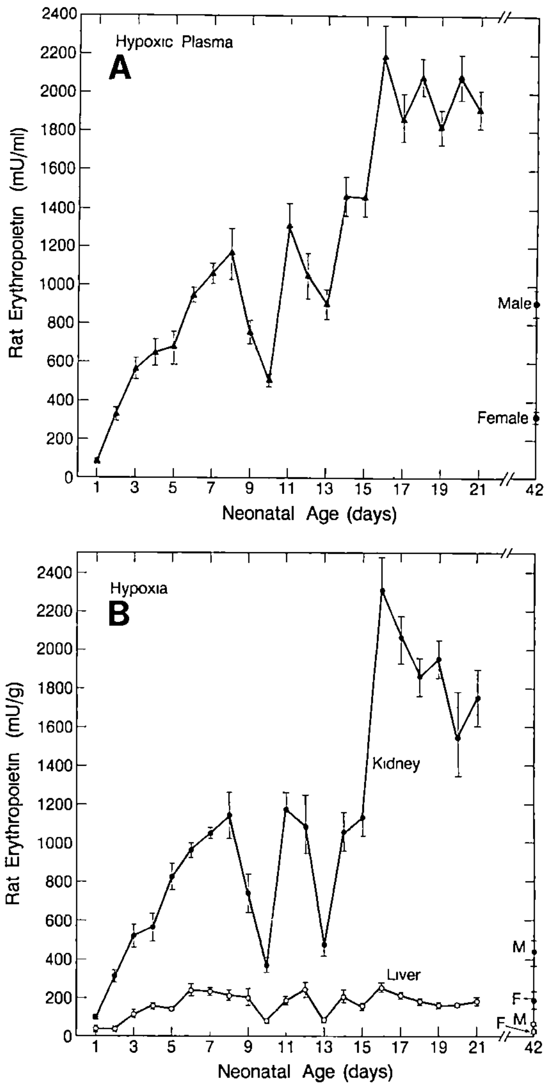

Fig 2. Erythropoietin concentrations in plasma (A) and kidney and liver (B) in the neonatal rat exposed to hypoxia (0.5 atm × 18 hours) as a function of age. Values are means ± SEM.

Lack of Measurable Erythropoietin in Rat Milk and Transfer to Neonates

No erythropoietin could be measured in milk from normal or hypoxic lactating rats (4 mU/mL). Similarly, no increases were measured in the plasma of pups that had been removed from their mothers overnight and returned in the morning after hypoxic exposure. Plasma values in neonates nursed by hypoxic mothers measured 60.7 ± 3.2 mU/mL (SEM, n = 7 pools of three pups each), and in those nursed by normoxic mothers were 58.6 ± 4.5 mU/mL (n = 6 pools of three pups each). Similarly, no differences were measured in liver and kidney tissue homogenates of these young rats. Because of the intraassay variation of approximately 9% there may still be some transfer from the mother to the pup via the milk, but the RIA is unable to detect this.

DISCUSSION

The data presented here show that erythropoietin can be measured in the rat during the last week of fetal life and that

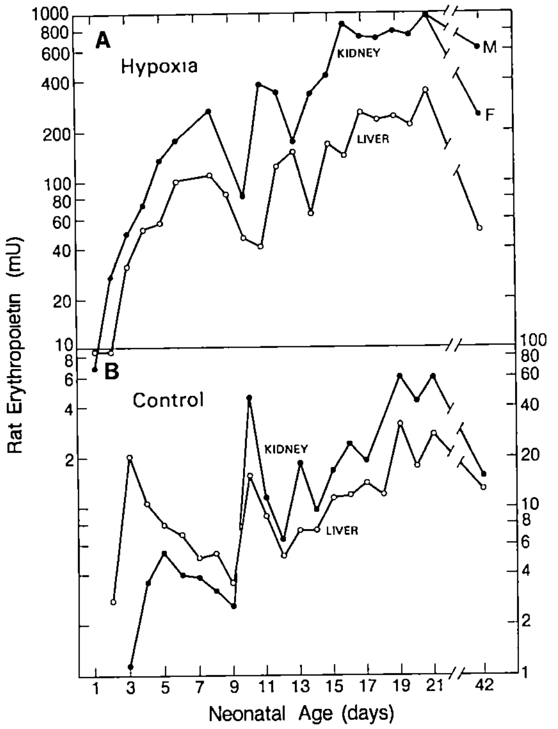

Fig 3. Total liver (○) and kidney (●) tissue erythropoietin content (mU/g × tissue weight) in the neonatal rat as a function of age under hypoxic (A) and normoxic (B) conditions. M, male; F, female.

the fetal response to maternal hypoxia is a function of age or, more specifically, a function of the developmental maturity of the Ep-producing tissues involved. The observation of relatively high levels of Ep in the AF and in the pups on day 14, and the fact that Ep levels in pups were still high on day 15 of gestation even though the liver with lower Ep levels had been removed, seems to suggest that in the fetal rat, and possibly also in other species, fetal hepatic Ep production is preceded by extrahepatic Ep synthesis that does not respond to maternal hypoxia. Early renal origin of Ep can be ruled out because from the first day the liver was removed (day 15), the early kidney tissue (either kidney anlage or pronephron) was also removed even though it could not be analyzed. It has been shown by Wells[24] that the fetal rat kidney is only able to secrete urine during the last 2 days of gestation and the elevated levels in AF on day 21 are most likely the result of increased renal Ep synthesis in response to maternal hypoxia. The importance of these relatively high levels of Ep at this stage of development is not understood. Cole and Paul[25] have reported that yolk sac erythropoiesis cannot be enhanced by exogenous Ep. The elevated levels in fetal rats could either be necessary for initiation of hepatic erythropoiesis until hepatic Ep synthesis can occur, or, more likely, Ep might have additional functions. It is possible that Ep acts as a growth factor alone or in conjunction with other growth factors in order to facilitate growth.

It is of interest that Ep levels and concentrations decline with advancing gestation except in renal tissue. This phenomenon occurred in the AF and pups and, apparently independently, in the fetal liver tissues and plasma during the last three days of gestation (irrespective of whether the mothers were exposed to hypoxia or not). There appear to be two possible explanations for this observation. The first possibility is that less Ep is needed because of increased sensitivity of the target tissue(s). This has been shown for somatomedins in fetal sheep where low levels were measured at a time of greatest need.[26] The other possibility might be that target tissue sensitivity has not changed, but rather that the erythropoietic system is already "turned on" and operating at the highest rate. Both situations would explain the fact that at birth the newborn rat has a reticulocyte count between 90% and 95%,[17,27] and therefore the decline in Ep at this time is unlikely to be the result of suppressed erythropoiesis.

In the newborn rat immunoreactive rat plasma and tissue Ep levels vary greatly during the first three weeks of life regardless of whether the animals were kept at ambient pressure or exposed to 18 hours of hypoxia. With the exception of the first and ninth days of life the circulating Ep levels were higher than adult levels in the normal newborn rat. The initial rise was probably attributable to hepatic Ep synthesis. From ten days of age renal Ep content was reflected in plasma levels. The liver continued to be a contributor up to days 10 and 11. The elevation in hepatic Ep content during this time cannot be due simply to residual blood content because a similar increase could not be measured on day 19 when plasma levels were equal to those observed on day 10.

Exposure of neonates to hypoxia confirms results reported earlier that neonatal rats respond to hypoxia with increasing Ep levels and that the response improves steadily with age.[6,9,10] In fact, during the third week of life, the levels measured in this study significantly exceeded those found in adult male rats exposed to the same hypoxic stimulus. Of the above cited studies only Gruber et al[9] found the levels in intact neonatal hypoxic rats to be higher than in adults when intact rats were tested at the age of 2 and 3 weeks. It appears that at this age an exposure of six hours at 0.35 atm may be comparable to 18 hours at 0.5 atm used in this study. Other authors found the response in the neonatal rat to be below that measured in adult animals, but this may be due to either a shorter time of exposure and/or less hypoxic stress.[6,10] From days 10 to 21 of age female and male rats responded equally to hypoxic exposure, in terms of renal Ep production and resulting circulating Ep levels. At the onset of maturity (day 42) the response was equal to that found in adult rats.

This present study neither could confirm the presence of Ep in milk obtained from normoxic and hypoxic mothers nor establish whether the Ep levels were elevated in the newborns nursed by hypoxic mothers. Lactating rats exposed to the same hypoxia usually have plasma levels of approximately 300 to 400 mU/mL, and we were not able to show any transfer from the mother to the young. The age of seven days was chosen for this particular experiment, because at this time endogenous renal and hepatic Ep levels were low, and the plasma levels were declining and still separated from the ten-day peak by three days (Figure 1 and Table 2). It also appears unlikely that Ep was inactivated by acid hydrolysis in the stomach because the pH in the neonatal rat stomach is reported not to fall below six before day 10,[28] and we have found that Ep becomes immunologically unreactive below pH 4.5. Our results appear to be in contrast to Carmichael et al[29] who observed increased erythropoiesis in neonates suckled by anemic mothers, and after oral Ep administration at ten days of age. We did not analyze peripheral blood and bone marrow values and increased erythropoiesis is not necessarily excluded here, even though Lucarelli et al[17] also could document no change in erythropoiesis in young rats nursed by normal or hypertransfused mothers.

Whether the neonatal rat derives most of its erythropoietin from the liver or the kidney has been the subject of investigation for years.[6,9,12,14,30] These studies showed that Ep production in nephrectomized newborn rats after hypoxia is only slightly reduced or not affected at all. Our data in intact rats suggest that the liver-to-kidney switch of Ep production in the normoxic neonatal rat occurred at day 10 and in the hypoxic newborn on day 2 of life. The conclusions reached by Gruber et al[9] and Lucarelli et al[17] that Ep production in significant measure is independent of renally produced Ep during the neonatal period in the rat, appears not to be correct when applied to the intact rat, and only true in the nephrectomized neonatal rat. The effects observed with nephrectomized rats were rather due to the fact that during the first 3 weeks of life, the liver retains the ability to synthesize Ep and fully compensates in the absence of the kidneys. This ability is mostly lost in the adult, although the liver has been found to be the principal site of extrarenal Ep production[31] and is able to maintain baseline quantities of Ep in the absence of the kidneys. Recent evidence has shown that in fully compensated hemolytic state induced in the rat by long-term phenylhydrazine administration the liver can become the primary source of Ep,[32] indicating that it is even possible to reverse the switch in the adult with time.

While it is important to be able to measure circulating plasma and tissue concentrations and determine the time of liver-to-kidney switch in the developing rat, no explanations can be offered at the present time to the following question. Why would an animal whose erythropoiesis can neither be stimulated further by exogenous Ep administration or hypoxia nor be totally suppressed by hypertransfusion[20] maintain higher-than-adult levels during the first three weeks of life, and during hypoxic conditions, elaborate even more than is necessary for the adult to reach erythropoietic homeostasis? This is especially puzzling because Miller et al[33] found that very small increases in the levels of Ep (approximately 5 mU/mL) are capable of significant increases in red blood cell production in the adult rat. The possibility has to be considered that the function(s) of Ep in neonatal rats may be multifold and not just be limited to erythropoiesis. Rather, Ep may work in conjunction with other growth factors until adult homeostasis is achieved, a fact that would also support the suggestion by Lucarelli et al[14,17] that erythropoiesis in the neonatal rat is governed differently from that in the adult.

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REFERENCES

1. Zanjani ED, Mann LI, Burlington H, Gordon AS, Wasserman LR: Evidence for a physiologic role of erythropoietin in fetal erythropoiesis Blood 44:285, 1974

2. Widness JA, Susa JB, Garcia JF, Singer DB, Sehgal P, Oh W, Schwartz R, Schwartz H. Increased erythropoiesis and elevated erythropoietin in infants born to diabetic mothers and in hyperinsulinemic Rhesus fetuses J Clin Invest 67:637, 1981

3. Phillips AF, Widness JA, Garcia JF, Raye JR, Schwartz R: Erythropoietin elevation in the chronically hyperglycemic fetal lamb. Proc Soc Exp Biol Med 170:42, 1982

4. Zanjani ED, Poster J, Burlington H, Mann LI, Wasserman LR. Liver as the primary site of erythropoietin formation in the fetus. J Lab Clin Med 89 640, 1977

5. Zanjani ED, Ascensao JL, McGlave P, Banissadre M: Studies on the liver to kidney switch of erythropoietin production J Clin Invest 67:1183, 1981

6. Carmena A, Howard D, Stohlman F: Regulation of erythropoiesis XXII Erythropoietin production in the newborn animal. Blood 32:376, 1968

7. Clemons GK, Garcia JF Immunoreactive erythropoietin concentrations in fetal and neonatal rats and the effects of maternal and neonatal exposure to hypoxia Exp Hematol 11:407, 1983 (Suppl 14) (abstr)

8. Haga P, Kristiansen S: Role of the kidney in fetal erythropoiesis and erythropoietin levels in newborn mice with renal agenesis J Embryol Exp Morphol 61:165, 1981

9. Gruber DF, Zucali JR, Wleklinski J, LaRussa V, Mirand EA: Temporal transition in the site of rat erythropoietin production. Exp Hematol 5 399, 1977

10. Caro J, Erslev AJ, Silver R, Miller O, Birgegard G: Erythropoietin production in response to anemia or hypoxia in the newborn rat. Blood 60:984, 1982

11. Meberg A: Plasma erythropoietin levels in fetal and newborn rats. Response to hypoxia. Exp Hematol 8:615, 1980

12. Schooley JC, Mahlmann LJ: Erythropoietin production in the anephric rat. I Relationship between nephrectomy, time of hypoxic exposure, and erythropoietin production Blood 39:31, 1972

13. Garcia JF: Erythropoietic response to hypoxia as a function of age in the normal male rat. Am J Physiol 190:25, 1957

14. Lucarelli G, Porcellini A, Carnevali C, Carmena A, Stohlman F Jr Fetal and neonatal erythropoiesis. Ann NY Acad Sci 149:544, 1968

15. Carmena AO, Lucarelli G, Carnevali C, Stohlman F: Regulation of erythropoiesis XIX. Effect of hypoxia on erythropoiesis in the newborn animal. Proc Soc Exp Biol Med 121:652, 1966

16 Zaizov R, Matoth Y: Regulation of erythropoiesis in the newborn rat. Isr J Med Sci 7:846, 1971

17. Lucarelli G, Howard D, Stohlman F Jr: Regulation of erythropoiesis. XV. Neonatal erythropoiesis and the effect of nephrectomy J Clin Invest 43:2195, 1964

18. Fried W, Barone-Varelas J, Barone T: The influence of age and sex on erythropoietin titers in the plasma and tissue homogenates of hypoxic rats Exp Hematol 5:472, 1982

19. Alippi RA, Giglio MJ, Rio ME, Bozzini CE: Influence of

growth rate on the ability of hypertransfusion induced plethora to suppress erythropoiesis in the growing rat. Exp Hematol 9.209, 1981

20. Sanengen T, Halvorsen S: Regulation of erythropoiesis during rapid growth. Br J Haematol 61·273, 1985

21. Garcia JF, Ebbe SN, Hollander L, Cutting HO, Miller ME, Cronkite EP: Radioimmunoassay of erythropoietin: Circulating levels in normal and polycythemic human beings. J Lab Clin Med 99·624, 1982

22 Clemons GK: Comparison of radioimmunoassay and bioassay of erythropoietin, in Zanjani ED, Tavassoli M, Ascensao JL (eds) Humoral and Cellular Regulation of Erythropoiesis. Spectrum, New York, 1986 (in press)

23. Rodbard D, Hutt DM. Statistical analysis of radioimmunoassay and immunoradiometric (labeled antibody) assays. A generalized weighted, iterative, least-squares method for logistic curve fitting, in Radioimmunoassays and Related Procedures in Medicine. Vienna, IAEA, p 165

24. Wells LJ. Observations on secretion of urine by kidneys of fetal rats. Anat Rec 94.504, 1946 (abstr)

25. Cole JR, Paul J The effects of erythropoietin on haem synthesis in mouse yolk sac and cultured fetal liver cells. J Embryol Exp Morph 15:245, 1966

26 Tato L, Marc VL, Prevot C, Rappaport R: Early variations of plasma somatomedin activity in the newborn J Clin Endocrinol Metab 40:534, 1975

27. Matoth Y, Zaizov R: Regulation of erythropoiesis in the fetal rat. Isr J Med Sci 7·839, 1971

28. Johnson LR: Functional development of the stomach. Annu Rev Physiol 47:199, 1985

29. Carmichael RD, Gordon AS, LoBue J: The effects of maternal phlebotomy and orally-administered erythropoietin (Ep) on erythropoiesis in the suckling rat. Biol Neonate 33.119, 1978

30. Schooley JC, Mahlmann LJ. Extrarenal erythropoietin production by the liver in the weanling rat Proc Soc Exp Biol Med 145.1081, 1974

31 Fried W: The liver as a source of extrarenal erythropoietin production. Blood 40.671

32. Dornfest BS, Naughton BA, Johnson R, Gordon AS: Hepatic production of erythropoietin in a phenylhydrazine-induced compensated hemolytic state in the rat. J Lab Clin Med 102:274, 1983

33. Miller ME, Garcia JF, Shiue GG, Okula RM, Clemons GK· Humoral regulation of erythropoiesis, in Killman SY-AA, Cronkite EP, Muller-Berat CN (eds). Haematopoietic Stem Cells. Copenhagen, Munksgaard, 1982, p 217

The Contribution of Cytochemistry and Immunophenotyping to the Reproducibility of the FAB Classification in Acute Leukemia

By George P. Browman, Peter B. Neame, and Praniti Soamboonsrup

Intraobserver and interobserver reproducibility of the FAB classification was assessed for two independent observers whose decisions are acted on for treatment of patients with acute leukemia in the Hamilton region. Intraobserver reproducibility was assessed for Wright-stained preparations that were examined independently on two consecutive occasions at least 2 weeks apart. A third reading was performed with Wright stain and cytochemical data, and the fourth reading was done with addition of immunophenotype data. Concordance was calculated using a statistic that corrects for chance-expected agreement (k), and a weighted statistic that takes into account the seriousness of disagreements was used. Samples were available for morphological and cytochemical assessment on 105 patients, and immunophenotype data were available on 93 specimens. Intraobserver concordance was 64.8% and 70.5% for observers A and B, respectively, with kappa values of .56 and .62. There were 37 discordant readings for observer A and 31 for observer B, with each observer discordant between lymphocytic:nonlymphocytic phenotypes in ten cases. Concordance between observers was 63% (k = .54) and 72% (k = .65) for each of two separate readings for Wright-stained preparations only. Reproducibility improved to 89% (k = .86) when cytochemistry was added. When immunophenotype information was provided in addition to Wright-stained and cytochemical preparations, the agreement was 99%. Lymphocytic:nonlymphocytic discordance between observers occurred on nine occasions when Wright-stained preparations only were available and four times when cytochemistry was added; it did not occur with immunophenotyping. The study suggests that immunophenotyping, when added to morphological assessment of acute leukemia, may contribute substantially to agreement between observers.

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THE FRENCH-AMERICAN-BRITISH (FAB) morphological classification for acute leukemia represents an important contribution toward a better understanding of this disease. Recent studies that demonstrate an association between morphological subtypes and specific chromosomal abnormalities provide evidence for the biological relevance of the classification.[1,2] The clinical relevance of the classification is well recognized. Treatment approaches markedly differ for patients with acute lymphocytic leukemia and those with acute nonlymphocytic leukemia. Certain subtypes of acute leukemia are associated with unique clinical problems for which specific therapeutic strategies are used (eg, anticoagulation to prevent disseminated intravascular coagulation in patients with promyelocytic leukemia). There is suggestive but as yet insufficient evidence of a relationship between FAB subtypes and prognosis.[3,4]

The morphological criteria for the FAB classification were developed from a consensus-seeking approach of a number of investigators and investigator panels.[4-7] An essential feature of any classification is that there be an acceptable level of reproducibility on repeated measurements within individuals and between individuals. A number of studies have examined how individuals agree with one another and with panels of experts on independent readings of slides from patients with acute leukemia. The studies reported to date have shown a substantial degree of agreement among observers: however, a number of important issues remain to be addressed. Although most studies of concordance have examined agreement between observers, or between panels of observers, few studies have explored concordance within observers. None of the studies has examined the pattern of within-observer discordance. The extent to which observers agree with themselves on separate examinations must be considered an important part of the formal evaluation of concordance.

None of the studies of concordance of the FAB classification has taken into account the agreement that would be expected to occur by chance. This is important, since the extent of agreement expected by chance will vary with the distribution of cases, the number of categories used for classification, and the number of observers. It is, therefore, difficult to compare extent of agreement across studies, since these variables differ. For most of the concordance studies that have been reported, the results have focused on the extent of agreement between individuals with panels of experts, or between panels of experts. In real practice, however, treatment decisions must often be made on the basis of a single individual's diagnosis without access to a panel of acknowledged experts.

In order to address these issues, we have performed a study to examine intraobserver and interobserver concordance for two observers whose decisions are acted on to plan induction therapies in acute leukemia. As part of this evaluation, we have examined how cytochemistry contributes to the degree of agreement between these observers. Furthermore, we have developed criteria for the classification of acute leukemia based on immunophenotyping[8] and have assessed the contribution of this system to overall concordance between observers. Concordance is expressed using a statistic that accounts

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for chance-expected agreement, and a weighted statistic is used that gives credit for partial agreement.

MATERIALS AND METHODS

Material for examination was assembled from samples submitted consecutively to the Regional Center between December 1983 and July 1985. To assess the reproducibility of the FAB classification and the contribution of cytochemistry, two experienced observers examined Wright-stained slides on two occasions at least two weeks apart. Two weeks later each observer independently reviewed the coded slides along with cytochemically stained specimens. A fourth reading was also done in which immunophenotyping information was provided in addition to the morphological and cytochemical data to determine how the observers used this information when coming to a final diagnosis.

FAB classification. The criteria used to established the FAB status of each sample are those reported by Bennett et al with minor modifications[4-8] The cytochemical criteria used to aid in FAB classification are those suggested by the FAB Co-operative Group.[5,7] Cytochemical stains used were periodic-acid Schiff, Sudan black, myeloperoxidase, chloroacetate esterase and nonspecific esterase, and acid phosphatase.[9,10]

Immunophenotyping The methods used have been described previously[10] and are further presented in a recent paper.[8]

Statistical considerations A nine-category classification scale was used by the observers: M-1, M-2, M-3, M-4, M-5, M-6, L-1, L-2, L-3.

Concordance was determined in three separate ways:

1. Percentage agreement was calculated as the proportion of the number of complete agreements among the total number of judgments.

2. The kappa statistic was used to express the extent of agreement over and above that expected by chance [11] The formula for kappa (k) is:

$$k = \frac{p_0 - p_c}{1 - p_c},$$

where p_0 = the observed proportion of agreements and p_c = chance-expected proportion of agreements.[11] Kappa has a value of 0 if the observed agreement equals chance-expected agreement; +1 if observed agreement is perfect; and <0 if observed agreement is less than chance-expected agreement.

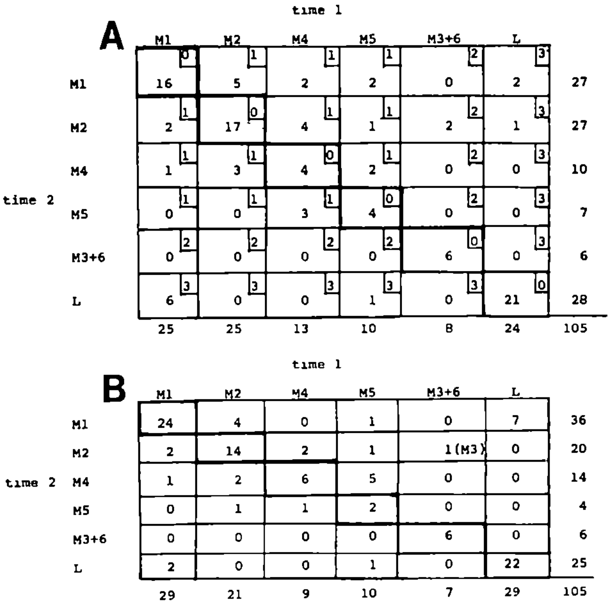
3 The weighted kappa statistic (k_w) was used to give credit for partial agreement, since not all disagreements are equally important [12] The assigned weights are based on the magnitude (or seriousness) of observed disagreements and are determined arbitrarily. The formula for k_w is:

$$k_w = \frac{1 - q_0}{q_c},$$

where q_0 = observed proportion of weighted disagreements and q_c = chance-expected proportion of weighted disagreements. As with kappa, k_w takes on values ranging from −1 to +1, with 0 representing chance-expected weighted agreement. For the purpose of this study, the following arbitrary weights were assigned 0, perfect agreement (no disagreement); 1, disagreement among M-1, M-2, M-4, M-5 or among L1-2, L-3; 2, disagreement between M-3 and M-1, M-2, M-4, M-5, or M-6 and disagreement between M-6 and M-1, M-2, M-3, M-4, or M-5; 3, disagreement between any M and any L

RESULTS

Intraobserver concordance. From December 1983 to July 1985, specimens from 105 consecutive patients were submitted for evaluation. Morphological evaluation with cytochemistry was performed on all specimens. Immunophenotyping data were available for 93 samples.



A

time 1

		M1	M2	M4	M5	M3+6	L	
time 2	M1	0 16	1 5	1 2	1 2	2 0	3 2	27
	M2	1 2	0 17	1 4	1 1	2 2	3 1	27
	M4	1 1	1 3	0 4	1 2	2 0	3 0	10
	M5	1 0	1 0	1 3	0 4	2 0	3 0	7
	M3+6	2 0	2 0	2 0	2 0	0 6	3 0	6
	L	3 6	3 0	3 0	3 1	3 0	0 21	28
		25	25	13	10	8	24	105

B

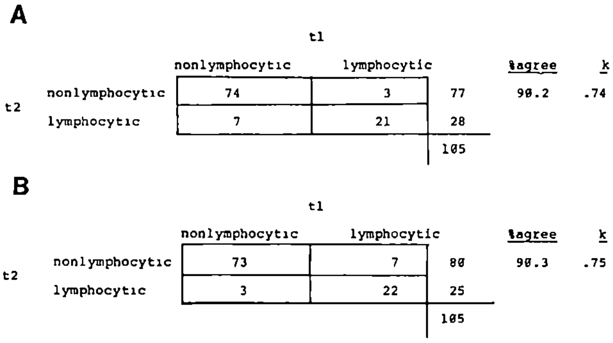
time 1

		M1	M2	M4	M5	M3+6	L	
time 2	M1	24	4	0	1	0	7	36
	M2	2	14	2	1	1(M3)	0	20
	M4	1	2	6	5	0	0	14
	M5	0	1	1	2	0	0	4
	M3+6	0	0	0	0	6	0	6
	L	2	0	0	1	0	22	25
		29	21	9	10	7	29	105

Fig 1. Agreement matrix within observers of a six-category scale for two separate readings, using Wright-stained preparations alone. (A) Observer A; (B) observer B. The weightings used for partial agreement are indicated in the upper right corner of each cell in (A).

Figure 1 shows the intraobserver agreement matrix for each observer for a six-category scale in which M3 and M6 were pooled and all lymphoid subtypes were pooled separately into single categories. The weightings for disagreement among categories, which were used to calculate the k_w statistic, are inserted in the right upper corner of each cell in Fig 1a for illustrative purposes.

The pattern of disagreement is an important variable, since clinical management will depend on the FAB subtype. This is most relevant for lymphocytic *v* nonlymphocytic leukemias, in which induction regimens differ substantially. Figure 2 shows the intraobserver lymphocytic:nonlymphocytic agreement matrix for observers A and B separately.



A

t1

		nonlymphocytic	lymphocytic		%agree	k
t2	nonlymphocytic	74	3	77	90.2	.74
	lymphocytic	7	21	28		
				105		

B

t1

		nonlymphocytic	lymphocytic		%agree	k
t2	nonlymphocytic	73	7	80	90.3	.75
	lymphocytic	3	22	25		
				105		

Fig 2. Agreement matrix within observers of a two-category scale (lymphocytic:nonlymphocytic) for two separate readings, using Wright-stained preparations alone. (A) Observer A; (B) observer B.

Each observer agreed 90% of the time as to whether the leukemia was lymphocytic or nonlymphocytic. For each observer, there were ten discordant readings. Reference to the agreement matrices of Fig 1 show that of the 20 lymphocytic:nonlymphocytic disagreements, 17 (85%) involved the FAB M-1 subtype.

Figure 3 shows intraobserver concordance for lymphoid subcategories (L-1 through L-3) of acute lymphocytic leukemia. For observer A, there was agreement on only 67.7% of cases (k = .41), whereas for observer B, the results were similar (62.5% agreement, k = .43). However, of the ten discordant readings for observer A, all represented lymphocytic:nonlymphocytic disagreements, whereas for observer B, of the 12 discordant readings, ten represented lymphocytic:nonlymphocytic disagreements. Therefore, of the 22 disagreements, only two represented errors within lymphoid subtypes. There was no disagreement between L-3, and L-1, 2.

Table 1 summarizes concordance results for intraobserver ratings for both observers using the six-category scale. When morphology alone is examined, the overall percentage of agreement for the first and second examinations is 64.8% for observer A and 70.5% for observer B. When chance-expected agreement is accounted for, the kappa value is .56 for observer A and .62 for observer B. The k_w scores are higher than the k scores, since the weightings give credit for partial agreement.

The relationship between the k score and the strength of agreement will be discussed subsequently. There are no universally accepted guidelines to determine an "acceptable" level of agreement.

Interobserver concordance. The agreement between observers was assessed for each of two separate readings using Wright-stained slides only, and for a third reading in which cytochemical information was added. Figure 4 shows the agreement matrix for each of two separate readings of FAB classification using the six-category scale for which morphological information alone was available, and Fig 5 shows the agreement matrix when cytochemical data were also provided.

A

	L1	L2	L3	other	
L1	4	0	0	0	4
L2	0	15	0	7	22
L3	0	0	2	0	2
other	0	3	0	0	3
	4	18	2	7	31

B

	L1	L2	L3	other	
L1	7	1	0	1	9
L2	1	11	0	2	14
L3	0	0	2	0	2
other	2	5	0	0	7
	10	17	2	3	32

Fig 3. Agreement matrix within observers of a four-category scale, using Wright-stained preparations alone. (A) Observer A; (B) observer B.

Table 1. Intraobserver Concordance for Morphological Assessment

	Agreement (%)	Kappa (k)	Weighted k (k_w)
Observer A	64 8	.56	.66
Observer B	70.5	.62	.70

To determine how the observers would use the classification based on immunophenotype, this information was provided to each separately accompanied by cytochemical and Wright-stained preparations. This is not a true test of concordance for the immunophenotype method, since immunophenotype preparations themselves were not assessed. However, the study does provide information on how these data are used by the observers.

The data presented in Table 2 summarize the results of interobserver agreement for all conditions. When Wright-stained preparations only were available, there was 63% agreement (k = .54) on the first reading and 72% (k = .65) on the second reading. When weightings were used to credit partial agreements, k_w was .63 on the first reading and improved to .71 on the second reading. This suggests the possibility of a learning effect from the first to the second reading. Although the number of discordant readings declined from the first to the second (39 to 29), the proportion of lymphocytic:nonlymphocytic discordant pairs was identical (31%) among the total number of disagreements for the first (12/39) and second (9/29) readings.

Figure 5 shows the pattern of concordance between observers when cytochemical information was provided.

A

Obs A

		M1	M2	M4	M5	M3+6	L	
Obs B	M1	15	8	1	1	1	3	29
	M2	3	13	4	1	0	1	22
	M4	0	2	5	2	0	0	9
	M5	0	1	3	6	0	0	10
	M3+6	0	0	0	0	7	0	7
	L	7	1	0	0	0	20	26
		25	25	13	10	8	24	105

B

Obs A

		M1	M2	M4	M5	M3+6	L	
Obs B	M1	22	6	2	0	0	6	36
	M2	0	17	2	0	1	0	20
	M4	2	3	6	3	0	0	14
	M5	0	0	0	4	0	0	4
	M3+6	0	1	0	0	5	0	6
	L	3	0	0	0	0	22	25
		27	27	10	7	6	28	105

Fig 4. Agreement matrix between two observers on two separate readings, using Wright-stained preparations alone. (A) First reading; (B) second reading. The assessments were done at least 2 weeks apart.

		Obs A					
		M1	M2	M4	M5	M3+6	L
Obs B	M1	20	2	0	0	1	1
	M2	3	17	0	0	0	0
	M4	0	0	12	1	0	0
	M5	0	0	0	11	0	0
	M3+6	0	1	0	0	5	0
	L	3	0	0	0	0	28

Fig 5. Agreement matrix between two observers for a six-category scale in which Wright-stained and cytochemical preparations were available for assessment.

Interobserver concordance improved from k = .65 in the absence of cytochemistry to .86 when cytochemical data were added, and k_w improved from .71 to .88 The availability of immunophenotyping was associated with almost perfect agreement (k = .99). The influence of cytochemistry and immunophenotyping on nonlymphocytic:lymphocytic disagreements is also shown in Table 2. When Wright-stained preparations alone were examined, there were nine occasions in which lymphocytic and nonlymphocytic subtypes were discordant. Addition of cytochemical information resulted in only four such disagreements, while immunophenotyping eliminated this problem. These data suggest that the observers depended primarily on the immunophenotype pattern when making a final judgment.

Although the demonstration of a high degree of reproducibility does not necessarily establish the validity of a classification, we decided to use the immunophenotyping results as a "gold standard" to determine the final phenotype among discordant readings involving the lymphocytic:nonlymphocytic categories. These data are shown in Table 3. Among the ten discordant readings by observer A when Wright-stained preparations only were available, eight are classified as lymphocytic and two are of nonlymphocytic origin. For observer B, among ten discordant readings, six were lymphocytic and four, nonlymphocytic. Interobserver discordance among lymphocytic:nonlymphocytic subtypes in Wright-stained preparations occurred 12 times on the first reading, of which nine were immunophenotyped as lymphocytic and three as nonlymphocytic. On the second reading, eight of the nine discordant readings immunophenotyped as lymphocytic and one as nonlymphocytic. There were only four lymphocytic:nonlymphocytic discordant readings with cytochemistry, and all immunophenotyped as lymphocytic.

In view of the high degree of concordance with immunophenotyping, we have characterized all of the 93 cases for which this information was available. Fifty-five patients had acute nonlymphocytic leukemia, 25 had acute lymphocytic leukemia, and 13 had mixed phenotypes of which 12 were mixed lineage and one had dual markers on the same cells.[10] The acute lymphocytic leukemias, including those with dual markers, could be subclassified into 15 CALLA positive, 12 T cell, 5 null cell, 2 B cell, and 1 pre-B cell. There was only one disagreement among 93 samples in which observer A classified a sample as M-1 and observer B classified the same sample as M-2. For all other samples, there was perfect agreement within the nine-category scale of the FAB classification.

Table 3. Immunophenotype Diagnosis for Lymphoid: Nonlymphoid Discordant Readings

Condition	No of Discordant Readings	Immunophenotype Diagnosis	
		Lymphoid	Nonlymphoid
Intraobserver			
Morphology Observer A	10	8	2
Observer B	10	6	4
Interobserver			
Morphology 1st reading	12	9	3
2nd reading	9	8	1
Cytochemistry	4	4	0

The almost perfect agreement with immunophenotyping depends on agreed-to specific objective criteria for subclassification. On this basis we can conclude that observers who accept these objective criteria for immunophenotyping will agree in most cases. The objective criteria used by our observers is the subject of a separate report.[8]

DISCUSSION

The appropriate treatment of acute leukemia is influenced by the type of disease, which is usually characterized by morphological assessment. This is especially relevant for the distinction between acute lymphocytic and nonlymphocytic leukemias, in which induction regimens are quite different. Apart from a choice of induction therapies, the intensity of postremission consolidation chemotherapy may be influenced if an association between FAB subtype and remission duration is found in future studies. There is evidence that the FAB classification may distinguish between leukemias that are biologically different, since correlations between FAB subtype and specific nonrandom chromosomal abnormalities have been shown.[1,2] In view of these considerations, it is important that the reproducibility of the FAB classification and of any additional diagnostic procedures be properly evaluated.

We have presented a formal evaluation of an agreement

Table 2. Contribution of Cytochemistry and Immunophenotyping to Concordance Between Two Observers (Six-Category Scale)

	Agreement (%)	k	k_w	N	No. of Discordant Readings: Total	No. of Discordant Readings: Lymphoid Myeloid
Morphology alone t1	63	.54	.63	105	39	12
t2	72	65	.71	105	29	9
Morphology + cytochemistry	89	.86	88	105	12	4
Morphology + cytochemistry + immunophenotyping	99	.99	.99	93	1	0

Table 4. Agreement Studies Using FAB Classification in Acute Leukemia

First Author	Year	Ref No	Data Base	No of Diagnostic Categories	No of Cases	Agreement (%)		Comments
						Intraobserver	Interobserver	
Whittacker	1979	13	M,C	6	278	—	45.7	ANLL only; 3 observers
Viana	1980	14	M	4	170	—	74.8–86.8	Acute lymphocytic leukemia only; 2 observers
						—	69.4	3 Observers
Mertelsmann	1980	3	M	11	228	—	69	2 Observer groups; modified FAB
Bennett	1981	4	M	3	100	—	60	Acute lymphocytic leukemia only
Head	1985	15	M,C	9	233	63.8,76	58.3–80	Individual classification
This study			M	6	105	64.8,70.5	72	Kappa and weighted kappa statistics used to correct for chance agreement
			M,C	6	105		86	
			M,I,C	9	93		99	

Concordance results using consensus are not included, since consensus is not associated with independent blind assessment. ANLL, acute nonlymphocytic leukemia; M, morphology (Wright stain); C, cytochemistry, I, immunophenotype

study between observers whose decisions in almost all cases influence patient management in our region. We have used a statistical procedure that accounts for chance-expected agreement. Furthermore, we have attempted to determine the contribution of cytochemistry and immunophenotyping to reproducibility of the FAB classification. Our data indicate that immunophenotyping may contribute substantially to the morphological classification of acute leukemia. In this study, biphenotypic leukemias were diagnosed only by the immunophenotype method.

The majority of studies that have examined the reproducibility of the FAB or a closely related classification in acute leukemia focused on the agreement between single observers and a panel of acknowledged experts (see Table 4). Only one study mentioned the extent of intraobserver concordance, although no details regarding the pattern of disagreement within observers was presented.[15] In the majority of cases, agreement of panels of experts or of three or more observers was examined. As Table 4 indicates, some of these studies examined extent of agreement using morphological criteria alone, while others included cytochemical evaluation. The number of categories that were used to determine extent of agreement differed among the studies. In view of these differences, it is difficult to compare the results across studies. Furthermore, none of the previously reported studies used a statistical analysis that accounts for chance-expected agreement.

There are no universally recognized guidelines for what represents an acceptable level of reproducibility for the morphological classification of acute leukemia. The results reported in this study are similar to those of other studies, but for the reasons given, the results are not directly comparable. It would be highly desirable if reproducibility were perfect. However, where subjective judgments play an important role in classifying disease, it is unrealistic to expect that all observers will act identically. Thus, classifications that depend on human interpretations are subject to wide variation, which can be considered part of the measurement error. The establishment of easily recognizable and interpretable objective criteria will contribute substantially to reducing this variability.

An estimate of the level of agreement that can be expected when judgments about clinical information are made by two observers has been reviewed by Koran.[16] None of the studies reviewed in Koran's report dealt with hematologic/morphological evaluation. Moreover, most of the data reviewed dealt with simple two-category judgments, such as whether a disease or clinical sign (on physical examination, radiologic examination, EEG, EKG, etc) was present or absent, or normal or abnormal. For these evaluations, kappa scores ranged from <.3 to .83.

Demonstration of reproducibility is not a sufficient condition for establishing validity. Two observers can agree on an event, but both may be wrong. In this case, reproducibility may be high, but the validity of the observations cannot be said to have been established. Although reproducibility is not a sufficient condition for establishing validity, the remarkable consistency of the immunophenotyping results in this study suggest that surface marker analysis of acute leukemia should be considered an important element in the classification of this disease. In view of our results, it is recommended that criteria for immunologic classification of acute leukemias be established among investigators. Proper evaluation of concordance for these methods will require further studies. In addition, further studies will be required to determine whether clinical decisions based on different methods of morphological classification of acute leukemia will improve treatment results.

REFERENCES

1. Fourth International Workshop on Chromosomes in Leukemia, 1982: Correlation between morphology and karyotype. Cancer Genet Cytogenet 11:275, 1984

2 Rowley JD Biological implications of consistent chromosome rearrangements in leukemia and lymphoma. Cancer Res 44:3159, 1984

3. Mertelsmann R, Thaler HT, To L, Gee TS, McKenzie S, Schauer P, Friedman A, Arlin Z, Cirrincione C, Clarkson B: Morphological classification, response to therapy, and survival in 263 adult patients with acute nonlymphoblastic leukemia. Blood 56:773, 1980

4. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton

DAG, Gralnick HR, Sultan C: The morphological classification of acute lymphoblastic leukemia. Concordance among observers and clinical correlations. Br J Haematol 47·553, 1981

5. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976

6 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

7. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med 103:626, 1985

8. Neame PB, Soamboonsrup P, Browman GP, Meyer RM, Benger A, Wilson WEC, Walker IR, Saeed N, McBride JA: Classifying acute leukemia by immunophenotyping: A combined FAB-immunologic classification of AML. Blood (in press)

9. Goldberg AF, Barka T: Acid phosphatase activity in human blood cells Nature 195:297, 1962

10 Neame PB, Soamboonsrup P, Browman G, Barr RD, Saeed N, Chan B, Pai M, Benger A, Wilson WEC, Walker IR, McBride JA: Simultaneous or sequential expression of lymphoid and myeloid phenotypes in acute leukemia. Blood 65:142, 1985

11 Cohen J: A coefficient of agreement for nominal scales. Educ Psychol Meas 20·37, 1960

12. Cohen J. Weighted kappa: Nominal scale agreement with provision for scaled disagreement or partial credit. Psychol Bull 70:213, 1968

13. Whittacker JA, Withey J, Powell DEB, Parry TE, Khurshid M· Leukemia classification: A study of the accuracy of diagnosis in 456 patients. Br J Haematol 41:177, 1979

14. Viana MB, Maurer HS, Ferenc C: Subclassification of acute lymphoblastic leukemia in children· Analysis of the reproducibility of morphological criteria and prognostic implications. Br J Haematol 44·383, 1980

15. Head DR, Savage RA, Cerezo L et al. Reproducibility of the French-American-British classification of acute leukemia: The Southwest Oncology Group experience. Am J Hematol 18.45, 1985

16. Koran LM: The reliability of clinical methods, data and judgements N Engl J Med 293:642, 1975

Multilineage Hematopoietic Growth Factor Interleukin 3 and Direct Activators of Protein Kinase C Stimulate Phosphorylation of Common Substrates

By Stuart W. Evans, Donna Rennick, and William L. Farrar

In order to investigate early signal transduction events in myeloid cells, the phosphosubstrates of an interleukin 3 (IL 3)-dependent cell line, FDC-P1, have been analyzed. Using synthetic diacylglycerol as a direct activator of the unique calcium–phospholipid-dependent phosphotransferase protein kinase C (PK-C) and genetically engineered homogeneous IL 3, we have demonstrated a common element to signal transduction events associated with these stimulants. One novel substrate, p68 (68,000 kd), was rapidly phosphorylated in either IL 3- or diacylglycerol-stimulated cells. The phosphorylation of p68 was dose-dependent, with both the physiological ligand and diacylglycerol inducing the same maximal level of phosphorylation. Phosphorylation of p68 occurred in a time-dependent manner analogous to previously described kinetics of PK-C subcellular redistribution in the FDC-P1 cell line. The p68 substrate was also phosphorylated in a cell-free system under conditions designed to activate PK-C. Phosphoamino acid analysis demonstrated that the p68 molecule phosphorylated in intact cells as well as in a calcium–phospho-lipid-dependent cell-free system was phosphorylated on threonine residues, not tyrosine. These data support the hypothesis that the activation of PK-C that occurs after IL 3-receptor interaction which leads to the rapid phosphorylation of cellular proteins is an important element of the signal transduction mechanism in FDC-P1 cells. We propose that phosphorylation of the p68 molecule is a physiochemical marker for the activation of PK-C in myeloid cells, in response to the growth-promoting physiological ligand.

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THROUGH THE INTERACTION between a population of progenitor cells and appropriate polypeptide growth factors, vertebrate organisms are able to regulate the production of heterogeneous blood cells with diverse functional activities In addition to the biological characterization of distinct peptide signals involved in hematopoiesis,[1-4] several of these glycoprotein growth factors have been purified to homogeneity and their cDNA nucleotide sequences determined.[2,5-10]

Interleukin 3 (IL 3) is one member of the polypeptide hematopoietic growth factors collectively referred to as colony-stimulating factors (CSF). The apparent diversity of biological activities expressed by this molecule in vitro has resulted in several alternative nomenclatures based on the biological assays used, including multi-CSF, P cell-stimulating factor (PCSF), burst-promoting activity (BPA), mast cell growth factor (MCGF), and hematopoietic cell growth factor (HCGF).[11-14] Synthesized by mitogen- or antigen-activated lymphocytes,[15,16] IL 3 appears to control the growth and differentiation of pluripotent stem cells, leading to the production of all the myeloid blood cell types. Unlike other growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), which act by stimulating cells to proliferate, the hematopoietic growth factors, including IL 3, appear to be essential also for growth and the maintenance of cellular viability.[17] Fibroblasts maintain a quiescent viability in the absence of EGF or PDGF, while hematopoietic cells will die in the presence of serum depleted of CSFs.

The true extent of the biological role of IL 3 in vivo remains obscure. Although IL 3 can stimulate erythroid colonies (BPA) in vitro, this growth factor cannot be detected in vivo even in severely anemic animals with greatly elevated levels of erythropoietin, another growth factor closely associated with erythropoiesis.[18] Also, attempts to modulate erythropoiesis or eosinophilopoiesis in vivo using IL 3 have been unsuccessful.[19] Even though the role of IL 3 in vivo is not clear, the fact that it has such a wide range of biological activities in vitro and that its mechanism of action appears to be distinct from those of other growth factors, such as EGF, PDGF, and FGF, suggest that it is important to investigate its mechanisms of action.

The reversible phosphorylation of proteins is now recognized to be one of the major mechanisms by which intracellular events can be controlled by hormonal stimuli.[20] Several distinct phosphorylating enzymes can be distinguished by their cofactor requirements, protein substrate specificity, or the specific amino acid moieties to which they transfer phosphate groups. Among the best characterized phosphotransferases are the cyclic adenosine monophosphate-dependent protein kinases (PK-A)[21] and the calcium–calmodulin phosphotransferase system.[22] Complementing the protein kinases are a group of dephosphorylating enzymes, protein phosphatases (see reference 23 for a review). Two phosphorylating systems have recently been closely associated with the growth and transformation of cells. Tyrosine kinase activity appears to be intrinsic to several retroviral transforming proteins (for review see reference 24) as well as cellular receptors for growth factors, such as EGF and PDGF.[25,26] The second protein phosphotransferase system recently associated with cell growth and transformation is protein kinase C (PK-C). PK-C is believed to mediate the effects of the tumor-promoting phorbol esters[27] as well as potentially mediate the signal transduction process of oncogenes that have reported tryosine kinase activity.[28,29] Phorbol esters physiochemically substitute for diacylglycerol, a hydrolysis product of phosphatidylinositol (PI), in the activa-

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tion of PK-C. Ligand-receptor interaction for a variety of hormones and neurotransmitters is thought to be the physiological signal initiating PI hydrolysis in the plasma membrane (for review see reference 30). Concomitant with PK-C stimulation seems to be its association with the inner surface of the plasma membrane.[31-33] The previous observation that IL 3 was able to induce the translocation of PK-C from cytosol to the cell membrane, a phosphotransferase system shared by phorbol esters,[34] stimulated our interest in the possible role of this enzyme in the growth and differentiation of hematopoietic cells. The studies presented in this report examine the substrates of protein phosphorylation induced by IL 3 in homogeneous factor-dependent myeloid cells. We report here that IL 3 stimulates phosphorylation of protein substrates identical to those stimulated by direct physiochemical activators of PK-C. These studies provide direct evidence that PK-C has a role in the mechanism of signal transduction that affects the physiological response to IL 3 and characterizes the biochemical substrates of PK-C in myeloid cells.

MATERIALS AND METHODS

Recombinant murine IL 3 and granulocyte-macrophage (GM)-CSF were produced by one of us (D.R.) at DNAX, as previously described.[9,10] 1-Oleyol-2-acetylglycerol (OAG) was purchased from Avanti Polar Lipids (Birmingham, Ala). 4β-Phorbol 12β-myristate 13α-acetate (PMA) was purchased from Sigma Chemical Co (St Louis). ^{32}P-orthophosphate 10 mCi/mL in 0.2 mol/L HCl was purchased from Dupont-New England Nuclear (Boston). Vitamin D_3 analog α-1,25 dihydroxyvitamin D_3 was provided by Dr M. Uskokovic, Hoffman La Roche (Nutley, NJ). All other chemicals were purchased from standard sources.

Maintenance and isotopic labeling of FDC-P1 cell line. Myeloid FDC-P1 cells[35] were maintained in RPMI 1640 (Advanced Biotechnologies, Silver Spring, Md), 0.1 mg/mL glutamine, 10% fetal calf serum (Hyclone, Logan, Utah), 10% WEHI-3 conditioned medium, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Culture conditions were 5% CO_2, 9% relative humidity, and 37 °C. Before use, FDC-P1 cells were washed free of serum and IL 3 with three consecutive washes, two in IL 3-free and one in serum–IL 3-free medium and incubated for two hours at 37 °C. FDC-P1 cells rested in this manner were not irreversibly committed to death and behaved in proliferation assays as well as untreated cells.

The cells were then washed free of phosphate with two changes of 10 mmol/L HEPES saline and resuspended in phosphate-free RPMI 1640 at 1 × 10^7 cells per milliliter. Thirty minutes later 0.5 mCi of ^{32}P-orthophosphate equilibrated with 20 mmol/L NaOH was added to each 1-mL suspension. Orthophosphate pools of cells were allowed to equilibrate for one hour at 37 °C.

Ligand stimulation of cells and extraction of detergent-soluble proteins. Cells (10^7/mL) were stimulated for 15 minutes (unless otherwise stated) with either a mock material (material prepared exactly as genetically engineered IL 3 and GM-CSF but without biologically active factor), rIL 3 2 × 10^4 U/mL, GM-CSF 10^5 CFU/mL, OAG 100 μg/mL, PMA 10 ng/mL. All materials were added at a final protein concentration of 2 μg/mL (adjusted with fatty acid-free bovine serum albumin [BSA]). Units of IL 3 were defined by a proliferation assay, one unit stimulating 50% maximum proliferation in 10,000 cells. After stimulation, cells were washed once with ice-cold complete RPMI 1640. Cells were lysed in 1% Triton X-100, 10 mmol/L Tris-saline pH 7.5, 10 mmol/L NaF, 10 mmol/L EDTA and kept on ice for 30 minutes. Insoluble material was removed by centrifugation at 10,000 g for ten minutes.

Preparation of particulate membrane and cytosol fractions of FDC-P1 cells and cell-free protein kinase C phosphorylation. Cells were washed free of serum (two washes in 10 mmol/L HEPES saline) adjusted to 1 × 10^9 cells per milliliter in homogenization buffer (20 mmol/L Tris-HCl pH 7.5, 2 mmol/L PMSF, 5 mmol/L EGTA, 2 mmol/L EDTA, 11% wt/vol sucrose). Cells were then broken in a Dounce homogenizer. Broken cell suspension was layered over a 41% sucrose cushion and centrifuged at 100,000 g for one hour. The cytosol fraction was removed from above the interface while the particulate membrane was removed from the interface. The particulate membrane was washed and pelleted by centrifugation at 100,000 g for one hour and finally resuspended in homogenization buffer. Membrane and cytosol fractions were frozen on dry ice and stored at −70 °C. The basic reaction buffer 20 mmol/L Tris-HCl pH 7.5, 75 mmol/L Mg^{++} was used in all reactions. Ca^{++} (0.5 mmol/L or 5 mmol/L), phospholipid (12 μg/mL phosphatidylserine, 0.4 μg/mL 1,2-diolein), EGTA and partially purified PK-C were included, as indicated in figure legends. Reactions carried out at 32 °C were started by the addition of adenosine triphosphate (ATP) adjusted from cold ATP and ^{32}P ATP (>5,000 Ci/mmol) to give a final concentration of 1 mmol/L at 7.5 to 10 × 10^6 cpm.

Phosphoaminoacid analysis. Slices of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), corresponding to the location of phosphorylated proteins identified by autoradiography, were placed in an extraction buffer, 20 mmol/L ammonium phosphate 1% SDS, 5% 2-mercaptoethanol (2-ME), homogenized and shaken at 37 °C for 48 hours. Extracted proteins were precipitated together with 10 μg of BSA as carrier protein in 20% trichloroacetic acid. The precipitates were washed, twice with ethanol ether (3:1) and once with ether alone, air-dried, and then hydrolyzed with 6 mmol/L hydrochloric acid for one hour at 110 °C. HCl was removed by heating under vacuum in a rotor evaporator. Single-dimension thin-layer electrophoresis was carried out at 1,500 V for 40 minutes, using cellulose thin-layer plates and pyridine:glacial acetic acid:water (10:100:1890) buffer pH 3.5. Phosphoaminoacids were identified using unlabeled standards located with ninhydrin.

Sample preparation and electrophoresis. Samples for single-dimension SDS-PAGE were adjusted to 0.66 mmol/L Tris-HCl pH 6.8, 10% wt/vol glycerol, 5% 2-ME, 2% SDS. Samples for nonequilibrating pH gel electrophoresis (NEpHGE) were solubilized in final concentration of 9.5 mmol/L urea, 2% NP-40, 2% ampholines (4:1 pH 5 to 7 : pH 3.5 to 10), 5% 2-ME. For SDS-PAGE 7.5% to 15% gradient, 7.5 mm thick separating gel with a 3% stacking gel were used. Samples were loaded into preformed slots and separation was carried out at 15 mA per gel. For NEpHGE, the sample was loaded at the anode end of a 6% acrylamide, 2% ampholine (pH 3.5 to 10) tube gel (2 mm ID × 120 mm L). Separation was carried out at 400 V for four hours. Second-dimension SDS-PAGE was essentially the same as single-dimension SDS-PAGE except that after focusing, the NEpHGs were equilibrated in stacking gel buffer 1% 2-ME for 2.5 hours and fixed above the SDS-PAGE with 1% agarose.

Detection of radiolabeled proteins. After electrophoresis, the gels were washed in methanol:water:acetic acid (5:5:1) for at least three one-hour washes. Gels were then rehydrated by washing in water, supported on filter paper, and dried (Biorad gel drier, Richmond, Calif). Autoradiography was carried out using Kodak XAR 5 film.

RESULTS

The extent of phosphorylation induced by the interaction of IL 3 with its receptor and the role of PK-C in this signal transduction was studied. In order to examine these questions, we selected the murine IL 3-dependent cell line

FDC-P1 developed by Dexter et al as our homogeneous target cell population.[35] Two nonpeptide chemical compounds, the tumor-promoter PMA and a synthetic analog of diacylglycerol 1-oleyol-2-acetylglycerol (OAG), are known to activate PK-C in intact cells both directly and physiochemically.[37] The protein phosphorylation patterns induced by these agents were compared with those induced by IL 3 (a growth factor essential for FDC-P1 survival), GM-CSF (a growth factor for which our FDC-P1 cell lines appears to have no biological response and that is unable to replace IL 3), and the tumor-promotor 1,25 dihydroxyvitamin D_3, an analog of vitamin D_3 that does not appear to activate PK-C. Figure 1 shows the phosphorylated substrates from intact cells after treatment for 15 minutes with different stimulating agents. An obvious increase in phosphorylation of two substrates, a 68,000-dalton molecule (p68) and a 20,000-dalton molecule (p20), was noted in IL 3-, OAG-, and PMA-stimulated cells but not in cells stimulated by GM-CSF or the vitamin D_3 analog. We also observed apparent dephosphorylation of two bands (110,000 and 45,000 daltons) in the IL 3-stimulated cells but not in cells treated with any of the other agents, including OAG and PMA. Densitometric scans (Fig 2) of a similar autoradiograph confirm the visual appraisal that the principal changes are (a) increased phosphorylation of the p68 and p20 in IL 3- and OAG-treated cells and (b) dephosphorylation of the p110 and p45 in only IL 3-treated cells. The scans also suggest that other less obvious changes in phosphorylation may be taking place. Comparison of the IL 3-treated cells with mock-stimulated cells (Fig 2A) reveals small changes in phosphorylation of molecules with molecular weights (mol wt) between 100,000 and 60,000 daltons. These changes include increased phosphorylation and dephosphorylation of molecules. OAG treatment (Fig 2B) also increased phosphorylation of several molecules; in fact, the OAG-induced phosphorylation of some of these molecules appeared greater than that induced by IL 3. The only qualitative difference between IL 3 treatment and OAG treatment appears to be dephosphorylation of p110 and p45.

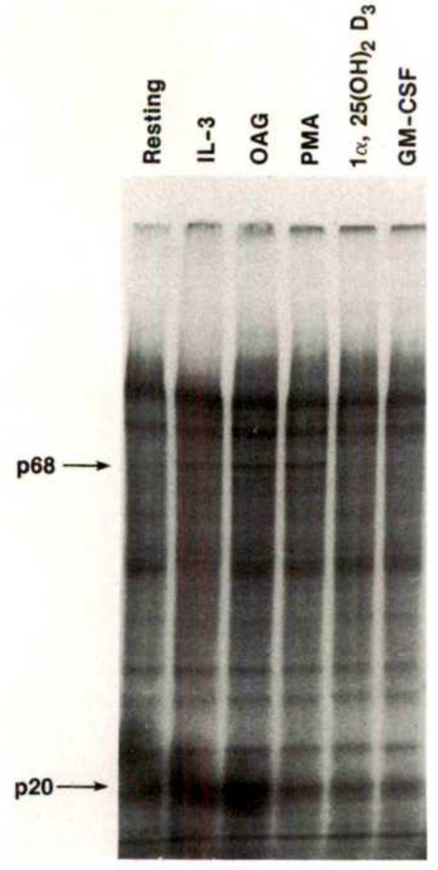

Fig 1. Single-dimension SDS-PAGE analysis of phosphosubstrates. Orthophosphate-labeled material extracted from (A) mock-, (B) IL 3-, (C) OAG-, (D) PMA-, (E) GM-CSF-, and (F) α-1,25 dihydroxyvitamin D_3-treated cells is compared. The positions of major differences are indicated as approximate molecular weights $\times 10^{-3}$ dalton.

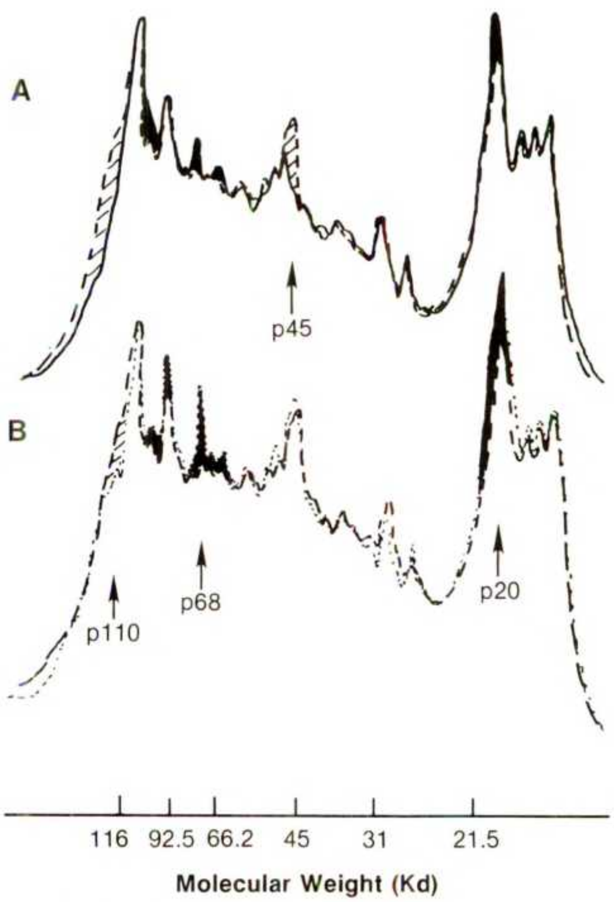

Fig 2. Densitometric scans of an autoradiograph from single-dimension SDS-PAGE analysis of phosphosubstrates from intact cells treated with either mock material ----, IL 3 ___, or OAG. . . . Scans show comparison between mock and IL 3 treatment (A) and mock and OAG treatment (B). Solid shade represents phosphorylation compared with control, while slashed shade represents dephosphorylation compared with control.

In order to determine better the extent of growth ligand-induced changes in phosphorylation, we compared the phosphoproteins by two-dimensional electrophoresis (Fig 3). The principal phosphorylation and dephosphorylation events associated with ligand stimulation are identified by com-

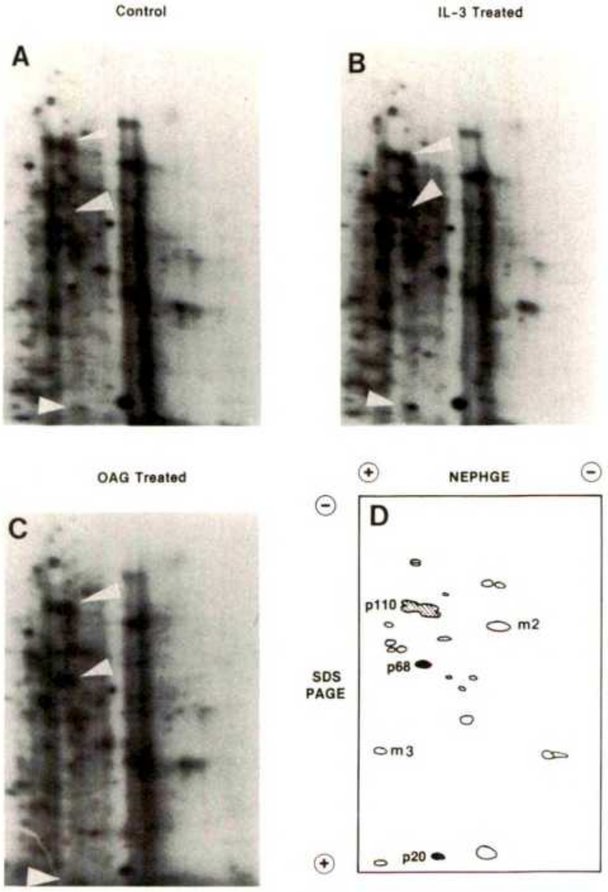

Fig 3. Two-dimensional NEpHGE × SDS-PAGE, O'Farral analysis of phosphosubstrates from intact cells. Orthophosphate-labeled material extracted from intact cells after treatment for 15 minutes with (A) mock material, (B) IL 3, and (C) OAG. Phosphosubstrates p68, p20, and p110, together with two reference proteins, M2 and M3 (M2 and M3 can be identified in Figs 5A and 6), are indicated by arrows and labeled on the map (D), with appropriate mol wt × 10^{-3} daltons. The anode and cathode for electrophoresis are also indicated on the map.

paring the phosphorylation patterns for control, mock-stimulated cells (3A), with IL 3-stimulated (3B) and OAG-stimulated (3C) cells. Both IL 3 and OAG stimulate phosphorylation of one molecule with identical charge-mol wt not detected in the resting cells. This molecule has a mol wt of approximately 68,000 daltons. A second molecule showing increased phosphorylation after stimulation of intact cells with either IL 3 or OAG has a mol wt of approximately 20,000 daltons. These molecules probably correspond to those identified by single-dimension electrophoresis. We were able to confirm the dephosphorylation of molecules with mol wt around 110,000 daltons induced by IL 3 but not by OAG. Therefore, two-dimensional analysis confirmed the principal changes detected in single-dimension analysis, at the same time extending the molecular properties compared. IL 3 and OAG induce phosphorylation of the same 68,000-dalton phosphoprotein, but IL 3 also induces dephosphorylation of a group of molecules with mol wt 110,000 daltons, while OAG increases the phosphorylation of a number of additional phosphoproteins. The principal changes observed were highly reproducible and could be identified by both single-dimension SDS-PAGE and two-dimensional NEpHGE × SDS-PAGE. Some of the smaller changes and the dephosphorylation of p45 that were identified by SDS-PAGE were less apparent. These changes were never seen on the two-dimensional phosphorylation patterns, suggesting that the p45 protein may be less abundant.

This study deals with phosphorylation changes in the FDC-P1 cell line. Using other IL 3-dependent cell lines, including FDC-P2, NSF 60, and NSF 60.8, we have observed that IL 3 stimulates the phosphorylation of a substrate with identical charge-mass ratio as p68. The analysis of p68 phosphorylation in these cells will be a subject of a separate paper that focuses on the ability of other growth factors, as well as IL 3, to activate PK-C. The 20,000-dalton protein phosphorylated by IL 3 or OAG stimulation comigrated with myosin light chain, a previously described PK-C substrate. Therefore, we chose to concentrate further studies on the uncharacterized 68,000-dalton molecule. Because the ligand-induced translocation of PK-C from cytosol to membrane is believed to be transient,[34] the temporal kinetic change in p68 phosphorylation associated with IL 3, OAG, and PMA stimulation of the FDC-P1 cells was analyzed. A time course analysis comparing the effects of IL 3, PMA, and OAG on phosphorylation of the 68,000-dalton molecule is represented in Fig 4A. The effect of IL 3 is transient, with peak phosphorylation at around ten minutes. Phosphorylation induced by OAG is also transient, while PMA-induced phosphorylation was more sustained and still significantly greater than background after two hours. Moreover, increases in relative phosphorylation were the result of increased ^{32}P incorporation into protein substrate, since protein levels of the 68,000 substrate did not vary during the experimental time course (data not shown). The phosphorylation pattern from an SDS-PAGE analysis illustrates the same result (Fig 4B). The changes induced by IL 3 appear transient; particularly clear is the change in phosphorylation of the 68,000-dalton molecule, while the effect of PMA was more persistent.

To demonstrate that the phosphorylation observed in intact cells was in fact due to PK-C, we carried out phosphorylation in a cell-free lysate enzyme reaction. In Fig 5A, phosphorylation of cytosolic and membrane fractions of FDC-P1 cells by a calcium–phospholipid-dependent kinase (PK-C) is shown. The reaction conditions described with the figure were designed to detect calcium–phospholipid-dependent PK-C substrates. In the cytosolic fraction, a group of calcium–phospholipid-dependent phosphosubstrates with mol wt of around 68,000 daltons was observed, while in the membrane fraction, a single dominant phosphosubstrate with mol wt of 68,000 daltons was detected. Analysis of the cytosolic (PK-C) phosphosubstrates by two-dimensional electrophoresis (Fig 5B) allows the identification of a molecule with identical charge-mass ratio as the 68,000-dalton substrate phosphorylated by either IL 3 or OAG in intact cells. The 68,000-dalton molecule seen in isolated membranes does not have the same charge-mass ratio (data not shown). It appears that the 68,000-dalton molecule that

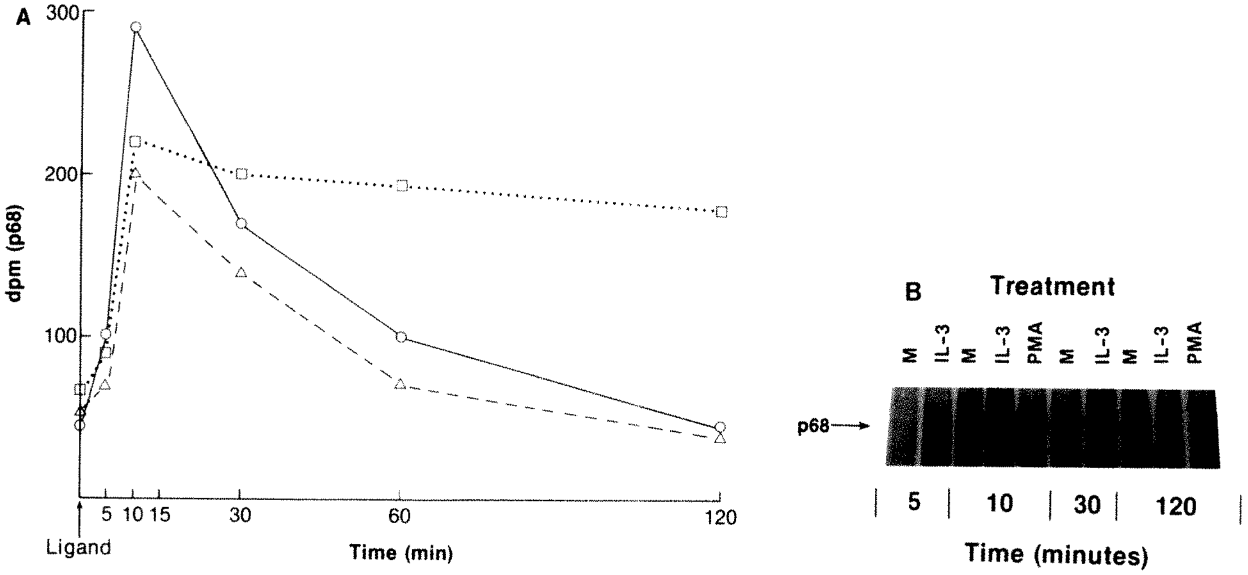

Fig 4. (A) Kinetics of p68 phosphorylation after treatment of cells with IL 3, OAG, or PMA. The 68,000-dalton molecule was excised from the gel and homogenized in 30% hydrogen peroxide for 30 minutes. The extent of Cerenkov radiation was then determined in a scintillation spectrometer. IL 3O——O, OAG Δ---Δ, PMA □ . . . □. (B) Single-dimension analysis of phosphosubstrates at different times after treatment of cells with mock material (M), IL 3, or PMA. Position of 68,000-dalton phosphosubstrate is indicated by arrow. Time since ligand stimulation (and corresponding to end point) is given below each pair of samples.

constitutes a minor phosphosubstrate for PK-C in cell-free systems is located in the soluble phase in intact cells, although a loose association with the plasma membrane cannot be excluded. Nevertheless, cell-free studies confirmed that the 68,000-dalton protein phosphorylated by IL 3 or OAG in intact cells was an enzymatic substrate of PK-C in vitro.

To confirm that the phosphorylation seen in intact cells was due to a serine-threonine rather than a tyrosine kinase, we carried out phosphoaminoacid analysis of the 68,000 PK-C substrate (Fig 6). It is clear that the phosphorylation of a 68,000-dalton protein in intact cells induced by either IL 3 or OAG occurs at threonine residues and not tyrosine.

DISCUSSION

Although many of the growth factors associated with the proliferation and differentiation of hematopoietic cells have been characterized and their biological activities established at least in vitro,[8-10] little is known about the mechanism of action of these molecules. We chose to begin our investigation of the molecular control of growth and differentiation by identifying the second-message systems affecting signal transduction immediately after ligand-receptor interaction. It has been suggested that phorbol esters potentiate mitogenesis through biochemical pathways that converge with those of hormones[37,38] and that the PK-C phosphotransferase system may be the one unifying site of this biochemical convergence.[39] With this in mind we set out to establish the role of PK-C as a signal transduction mechanism associated with the biological action of IL 3. By comparing the phosphorylation induced by agents that are known to directly activate PK-C with the phosphorylation seen after IL 3 stimulation of intact cells, we identified two obvious phosphosubstrates with the greatest net change in phosphorylation, p68 and p20, after either treatment with IL 3 or direct PK-C activators. This observation reinforces our previous findings that PK-C translocates to the plasma membrane after the interaction of IL 3 with its receptor. Phorbol esters, such as PMA, have been shown to induce the rapid translocation of PK-C from cytosol to membrane; concomitant with this translocation of PK-C the enzyme is activated.[31-34,40] We describe in this paper the phosphorylation events observed in the FDC-P1 cell line. The IL 3- and OAG-stimulated phosphorylation of one major substrate, p68, has been observed in other IL 3-dependent cell lines, FDC-P2 and NSF-60, suggesting that this substrate may be an important element in IL 3 signal transduction. Furthermore, we and others have identified a substrate with identical charge-mass ratio to p68 in cytotoxic T lymphocytes, which is phosphorylated after IL 2 and OAG stimulation of these cells.[41-44] These findings indicate that p68 may be highly conserved among various lineage cells and is coupled to other growth factor receptors, such as IL 2. Using the FDC-P1 cell line, we have previously shown that IL 3 will induce a rapid but transient translocation of PK-C from the cytosol to the membrane.[34] Interestingly, the transient phosphorylation of p68 we described in this study appears to parallel the kinetics of PK-C subcellular redistribution that we previously reported,[34] thereby providing further evidence that the PK-C phosphotransferase system is physiologically significant for transduction of the signal delivered by IL 3-receptor interaction. Differences primarily in the dephosphorylation pattern between the physiological ligand and OAG were also observed. IL 3 stimulated the dephosphorylation of p110 and p45 substrates, whereas OAG did not. This observation suggested the possibility of an additional receptor-coupled phosphoprotein regulatory system induced by IL 3 that is not stimulated by direct activators of PK-C. Because most physiological growth

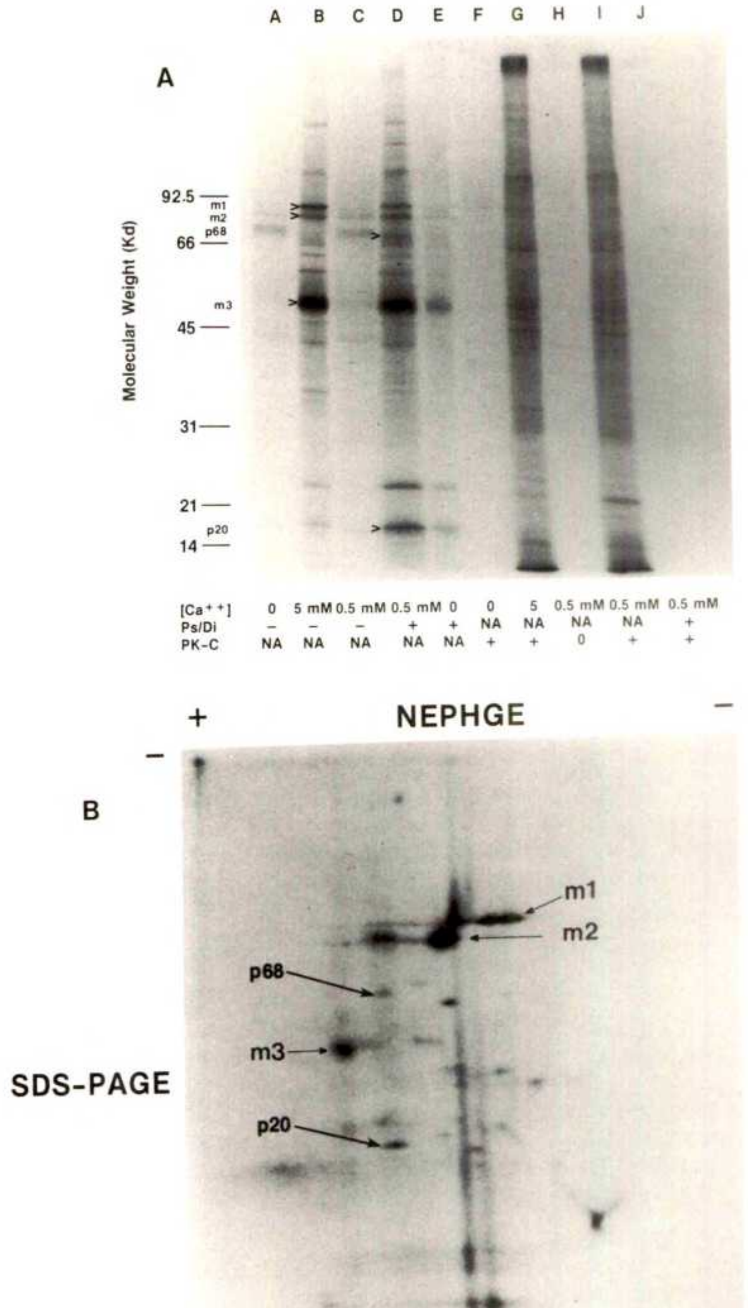
A
A B C D E F G H I J
Molecular Weight (Kd)
92.5
m1
m2
p68
66
m3
45
31
21
p20
14
[Ca⁺⁺] 0 5 mM 0.5 mM 0.5 mM 0 0 5 0.5 mM 0.5 mM 0.5 mM
Ps/Di – – – + + NA NA NA NA +
PK-C NA NA NA NA NA + + 0 + +
B
NEPHGE
+
–
–
+
SDS-PAGE
m1
m2
p68
m3
p20

Fig 5. **(A) Single-dimension SDS-PAGE analysis of cell-free phosphosubstrates generated under different calcium ion and phospholipid conditions. A-E cytosol, F-I membrane fraction prepared as described in Materials and Methods. Reaction conditions, indicated on the figure, were (A) cytosol, without Ca⁺⁺ or phospholipid; (B) cytosol, 5 mmol/L Ca⁺⁺; (C) cytosol, 0.5 mmol/L Ca⁺⁺; (D) cytosol, 0.5 mmol/L Ca⁺⁺ and phospholipids; (E) cytosol and phospholipid, no Ca⁺⁺; (F) membrane, PK-C, no Ca⁺⁺; (G) membrane, 5 mmol/L Ca⁺⁺; (H) membrane, no PK-C, 0.5 mmol/L Ca⁺⁺; (I) membrane, 0.5 mmol/L Ca⁺⁺ and PK-C; (J) 0.5 mmol/L Ca⁺⁺, phospholipid, and PK-C. References proteins M1, M2, and M3, as well as the p68 and p20 phosphosubstrates, are identified with arrow heads. (B) Two-dimensional NEpHGE × SDS-PAGE analysis of cell-free phosphosubstrates from a reaction similar to Fig 6, lane D. Cytosolic substrates incubated in the presence of calcium phospholipid and endogenous PK-C. The 68,000- and 20,000-dalton substrates are indicated, as well as three references proteins (M1, M2, and M3) identified on the SDS-PAGE (Fig 5A).**

ligands also stimulate calcium mobilization and direct stimulants of PK-C (OAG and PMA) do not, additional enzymatic systems regulated by the physiological ligand may involve calcium.

The inability of PMA and OAG to stimulate proliferation of the FDC-P1 cells is not surprising. First, other phosphorylation events not associated with the activation of PK-C, including the dephosphorylation of p110 as well as the other changes detected by densitometric scanning (Fig 2) may represent biochemical events distinct from PK-C. Second, even if physiological activation of PK-C is responsible for control of proliferation, the use of synthetic agents outside of normal feedback control might be expected to result in discordant biochemical events after long-term stimulation. The value of these pharmacological agents is in the short-term experiment in which the stimulation of rapid biochemical events mimics the activation of IL 3 on its receptor.

There is reasonable evidence that for some growth factors, such as EGF and PDGF, the respective receptors possess tyrosine kinase activity.[25,26] Accordingly, we examined

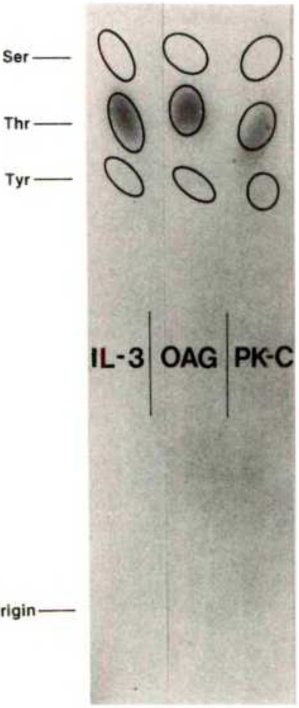

Fig 6. Phosphoaminoacid analysis of the 68,000-dalton molecule extracted from two-dimensional NEpHGE × SDS-PAGE. Using thin-layer electrophoresis to analyze products of the acid hydrolysis, the p68 phosphorylated in intact cells after IL 3 or OAG treatment together with the substrate phosphorylated by PK-C in a cell-free system are compared. The positions of cold phosphoaminoacid standards for serine threonine and tyrosine are marked as determined by development with ninhydrin.

whether the unique phosphosubstrates induced by IL 3 were phosphorylated at tyrosine or serine-threonine residues. The p68 protein phosphorylated in intact cells by IL 3 or OAG treatment and in a cell-free system was phosphorylated on threonine residues, not tyrosine. These results suggest that the phosphorylation of p68 is affected by the calcium-phospholipid-dependent PK-C but do not exclude the possibility that a more complex enzyme cascade has been effected to achieve phosphorylation of p68 by an enzyme regulated by PK-C. It is clear, however, that p68 is not being phosphorylated by a tyrosine kinase. It is interesting to speculate on the role of p68; a molecule of this size is known to be associated with the Na$^+$-H$^+$ anion exchanger in red cell membranes, and a similar molecule has been described in lymphocytes.[45] In view of the role IL 3 appears to have in maintaining the ATP level of FDC-P1 IL 3-dependent cells[46] and the linkage of ATP generation with the Na$^+$-H$^+$ anion exchanger, we suggest the possibility that p68 may be either a regulatory subunit associated with the cytosolic domain of an anion exchanger or, alternatively, a component of a phosphorylation cascade associated with the regulation of the Na$^+$-H$^+$ antiporter. However, other candidates for the p68 substrate exist, as suggested by the findings of a similar nuclear lamina phosphoprotein that appears to play a critical role in CHO cell proliferation and has a mol wt of around 68,000 daltons,[47] whereas in neural cells, a 70,000-dalton phosphoprotein has been identified, which is associated with the K$^+$ channel thought to be closely associated with depolarization of the cell membrane.[48]

We have demonstrated that PK-C is involved in the transduction of the signal delivered to cells by IL 3, by the identification of common phosphosubstrates. However, IL 3 induced specific changes in the phosphorylation pattern that activators of PK-C did not mimic. Most noticeable and reproducible of these changes was the dephosphorylation event seen in the 110,000-dalton range. Whether this dephosphorylation is due to a protein phosphatase associated with ligand signal transduction or simply reflects changes in molecule solubility after ligand-receptor interaction is under investigation. The finding that additional changes in protein phosphorylation occur with IL 3 stimulation obviously supports the notion that PK-C activation is part of a coordinated signal transduction process that may be evoked by the physiological ligand. Clearly, a role for calcium has been suggested as an additional modulating signal on hormone-receptor interactions.

In conclusion, the data demonstrate that the interaction of IL 3 with its receptor induces the rapid transient translocation[35] and activation of PK-C, which results in the reproducible phosphorylation of unique substrates also phosphorylated by direct activators of PK-C. IL 3 induced additional changes that were not observed when cells were treated with direct activators of PK-C, such as phorbol esters, suggesting that additional phosphoregulatory elements may be involved in the physiological signal transduction system.

REFERENCES

1. Stanley ER, Heard PM: Factors regulating macrophage production and growth. Purification and some properties of the colony-stimulating factor from medium conditioned by mouse L-cells. J Biol Chem 252:4305, 1977

2. Clark-Lewis I, Schrader JW: Biochemical characterization of regulatory factors derived from T cell hybridomas and spleen cells. I. Separation of T cell-growth factor and T cell-replacing factor from granulocyte-macrophage colony-stimulating factor. J Immunol 128:168, 1982

3. Iscove NN, Roitsch CA, Williams N, Guilbert LJ: Molecules stimulating early red cell, granulocyte, macrophage and megakaryocyte precursors in culture: Similarity in size hydrophobicity and charge. J Cell Physiol Suppl 1:65, 1982

4. Horiuchi M, Ichikawa Y: Control of macrophage and granulocyte colony formation by two different factors. Exp Cell Res 110:79, 1977

5. Burgess AW, Camakaris J, Metcalf D: The purification and properties of colony stimulating factor from mouse lung-conditioned medium. J Biol Chem 252:1988, 1977

6. Clark-Lewis I, Schrader JW, We Y-Y, Harris AW: A B lymphoma cell line produces growth factors for haemopoietic, lymphoid and mast cells. Cell Immunol 69:196, 1982

7. Taniguchi T, Matsui H, Jujita T, Takaoka C, Kashima N, Yoshimoto R, Manuro J: Structure and expression of a cloned cDNA for human interleukin 2. Nature 302:305, 1983

8. Fung MC, Hapel AJ, Yamer S, Cohen DR, Johnson RM, Campbell HD, Young IG: Molecular cloning of cDNA for murine interleukin 3. Nature 307:233, 1984

9. Yokota T, Lee F, Rennick D, Hall C, Arai N, Mosmann T, Nabel G, Canter H, Ari K: Isolation and characterization of a mouse cDNA clone that expresses most cell growth factor activity in monkey cells. Proc Natl Acad Sci USA 81.1070, 1984

10 Lee F, Yokota T, Otsnka T, Gemmell L, Larson N, Luh J, Arai K, Rennick D· Isolation of cDNA for a human granulocyte macrophage colony stimulating factor by functional expression in mammalian cells. Proc Natl Acad Sci USA 82·4360, 1985

11 Dexter TM: Blood cell development. The message in the medium. Nature 309 746, 1984

12. Clark-Lewis I, Schrader JW: P cell-stimulating factor. Biochemical characterization of a new T cell-derived factor. J Immunol 127:1941, 1981

13. Yung Y-P, Eger R, Tertian G, Moore MAS· Long-term in vitro culture of murine mast cells II. Purification of mast cell growth factor and its dissociation from TCGF. J Immunol 127:794, 1981

14. Burgess AW, Metcalf D, Russell SHM, Nicola NA: Granulocyte-macrophage, megakaryocyte, eosinophil- and erythroid-colony stimulating factors produced by mouse spleen cells. Biochem J 185·301, 1980

15 Schrader JW, Lewis SJ, Clark-Lewis I, Culvenor JG The persisting (P) cell histamine content regulation by a T cell derived factor, origin from a bone marrow precursor and relationship to mast cells. Proc Natl Acad Sci USA 78.323, 1981

16. Schrader MH, Iscove NN Haematopoietic growth factors are released in cultures of H-2 restricted helper T cells, accessory cells and specific antigens Nature 287:228, 1980

17. Nicola NA, Vadas M· Haemapoietic colony stimulating factors Immunol Today 5 76, 1984

18 Sherwood JB, Goldwasser E: A sensitive radioimmunoassay for erythropoietin Blood 54:885, 1979

19 Burgess AW: Hematopoietic growth factors, in Ford RJ, Maizel CA (eds). Mediators in Cell Growth and Diffentiation. New York, Raven, 1985, p 159

20. Cohen P: The role of protein phosphorylation in normal and hormonal control of cellular activity Nature 296.613, 1982

21. Rosen OM, Krebs EG (eds): Protein Phosphorylation. Cold Spring Harbor Conference on Cell Proliferation Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, vol 8, 1981

22. Manalan AS, Klee CB: Calmodulin, in Greengard P, Robinson GA (eds)· Advances in Cyclic Nucleotide and Protein Phosphorylation Research New York, Raven, 1984, p 227

23. Embi N, Parker PJ, Cohen P. The protein phosphatases involved in cellular regulation Eur J Biochem 115·405, 1981

24. Hunter T, Sefton BM Protein kinase and viral transformation, in Cohen PS, Van Heyningen S (eds). The Molecular Actions of Toxins and Viruses. Amsterdam, Elsevier/North Holland, 1982, p 333

25. Buhrow SA, Cohen S, Staros JV. Affinity labeling of the protein kinase associated with the epidermal growth factor receptor in membrane vesicles from A431 cells. J Biol Chem 257:4019, 1982

26. Ek B, Westermark B, Wasterson A, Heldin CH: Stimulation of trypsin-specific phosphorylation by platelet-derived growth factor Nature 295:419, 1982

27. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y: Direct activation of calcium activated, phospholipid dependent, protein kinase by tumor-promoting phorbol esters J Biol Chem 257.7987, 1982

28 Sugimoto Y, Whitman M, Cantle LC, Erikson LR Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositide and diacylglycerol. Proc Natl Acad Sci USA 81.2117, 1984

29. Macara IG, Marinetti GV, Balduzzi PC: Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity. Possible role in tumorigenesis. Proc Natl Acad Sci USA 81·2728, 1984

30. Berridge MJ, Irvine RF: Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 312·315, 1984

31. Kraft AS, Anderson WB, Cooper HL, Sando JJ: Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL 4 thymoma cells. J Biol Chem 257·193, 1982

32. Sando J, Young MC. Identification of high-affinity phorbol ester receptor in cytoxol of EL4 thymoam cells: Requirement for calcium, magnesium, and phospholipids. Proc Natl Acad Sci USA 80.2642, 1983

33 Kraft AS, Anderson WB: Phorbol esters increase the amount of Ca^+ phospholipid-dependent protein kinase associated with plasma membrane. Nature 301·621, 1983

34. Farrar WL, Thomas TP, Anderson WB· Altered cytosol/membrane enzyme redistribution on interleukin 3 activation of protein kinase C Nature 315:235, 1985

35. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D: Growth of factor-dependent hemopoietic precursor cell lines J Exp Med 152.1036, 1980

36 Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikua M, Nishizuka Y. Synergistic functions of proteins phosphorylation and calcium mobilization in platelet activation J Bio Chem 258·6701, 1983

37. Dicker P, Rozengurt E. Phorbol esters and vasopressin stimulate DNA synthesis by a common mechanism. Nature 287.607, 1980

38. Dicker P, Rozengurt E Stimulation of DNA synthesis by transient exposure of cell cultures to TPA or polypeptide mitogen. Induction of competence or incomplete removal? J Cell Physiol 109.99, 1981

39. Nishizuka Y: The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308:693, 1984

40 Anderson WB, Salmon D Calcium phospholipid and cellular proliferation, in Kud JF (ed): Phospholipids and Cellular Regulation Boca Raton, Fla, CRC Press, 1985, p 287

41. Evans SW, Farrar WL: Identity of common phosphoprotein substrates stimulated by interleukin 2 and diacylglycerol suggest a role of protein kinase C for IL 2 signal transduction. (submitted for publication)

42. Farrar WL, Cleveland JL, Beckner SK, Bonvini E, Evans SW· Biochemical and molecular events associated with interleukin 2 regulation of lymphocyte proliferation, in Moller G (ed). Immunological Reviews Transplantation Reviews, vol 92. Copenhagen, Munksgaard, July 1986

43. Farrar WL, Evans SW, Ruscetti FW, Bonvini E, Young HW, Birchenall-Sparks MC· Biochemical mechanism(s) of interleukin 2 regulation of lymphocyte growth, in Oppenheim JJ, Jacobs DM (eds): Leukocytes and Host Defense. New York, Alan R. Liss, 1986, p 75

44. Ishii T, Sugamura K, Nakamura M, Hinuma Y· Interleukin 2 (IL 2) rapidly induces phosphorylation of a cellular protein, p 67, in an IL 2 dependent murine cell line Biochem Biophys Res Commun 135 487, 1986

45 Owens RJ, Crumpton MJ. Isolation and characterization of a novel 68,000 Ca^{++} binding protein of lymphocytes plasma membrane. Biochem J 219:309, 1984

46. Wheton AD, Dexter TM: Effects of haematopoietic cell growth factor in intracellular ATP levels. Nature 303:629, 1983

47. Gerace L, Blobel G: The nuclear envelope lamina is reversibly depolymerized during mitosis. Cell 19.277, 1980

48. Lomas JR, Novak-Hoper I, Leritan IB· Phosphoproteins associated with the regulation of a specific potassium channel in the identified aplysia neuron RI5. J Biol Chem 260·3207, 1985

Variant Chronic Granulomatous Disease: Modulation of the Neutrophil Defect by Severe Infection

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Jonathan Wright, Orah S. Platt, Elizabeth R. Simons, and Alfred I. Tauber

The present studies document the cellular and biochemical processes involved in granulocyte O_2^- production in three patients from two kindreds with variant chronic granulomatous disease (CGD). Rates of O_2^- production were 9% to 30% of normal, depending on the individual tested and the stimulus; the two brothers from one family responded to each stimulus with rates very similar to each other. Kinetic analysis of NADPH-dependent O_2^- production in subcellular fractions revealed all three to have NADPH oxidases with both diminished substrate affinity for NADPH (high K_m^{app}) and decreased maximal velocities of O_2^- production. Their granulocytes had normal lag times for activation of the respiratory burst but abnormal rates of stimulus-induced membrane depolarization. Cytochrome b was not found in granulocytes or subcellular fractions despite the use of a spectrophotometric assay sensitive enough to detect the cytochrome if its content were proportional to the residual rate of O_2^- generation. A striking finding in one patient from each kindred was a threefold to tenfold decrease in the rate of O_2^- production accompanying serious infection. The residual O_2^--generating activity of CGD variants helps to explain their relative freedom from the recurrent infections of the classic disease. However, the marked decrease described in the present study indicates the potential for a vicious cycle in which an infection, once established, leads to increasing impairment of host defense.

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CHRONIC GRANULOMATOUS disease (CGD) is a hereditary disorder characterized by recurrent pyogenic infections that may lead to death in childhood.[1,2] Phagocytes from these patients fail to kill ingested microorganisms because of an inability to produce superoxide (O_2^-) and related toxic oxygen metabolites.[3,4] In normal phagocytes, a variety of particulate and soluble stimuli induces a rapid depolarization of the plasma membrane accompanied or followed by activation of a membrane-bound NADPH oxidase that reduces molecular oxygen to O_2^-.[4,5] Phagocytes from most CGD patients show neither membrane depolarization nor NADPH oxidase activity[6-9] and hence produce no detectable O_2^- [3] CGD is inherited in X-linked or, more rarely, autosomal recessive forms[10]; in the former, but not the latter, phagocytes lack cytochrome b_{-245}, a low midpoint potential cytochrome associated with NADPH oxidase.[11-13] Alternatively, a flavoprotein component may be absent.[14]

In addition to these classic forms of CGD, several variants have been reported, all with decreased but detectable neutrophil O_2^- production.[15-19] Most of these patients presented later in life with milder clinical disease than classic CGD patients. Their neutrophils showed normal[15,16] or only moderately decreased[17] membrane depolarization responses and altered NADPH oxidase kinetics consisting of an elevated Michaelis constant (K_m^{app}, reflecting decreased affinity for NADPH) with[16] or without[15,17,18] a decreased maximum velocity (V_{max}). In two of the X-linked cases[15,16] cytochrome b_{-245} was undetectable, but in one X-linked case[19] and in the autosomal recessive case,[17] the cytochrome content was normal.

The present study describes two additional kindreds with variant CGD. Both are X-linked, with absent cytochrome b_{-245} and near-normal membrane depolarization responses. Two affected patients showed striking, but reversible, decreases in O_2^- generation, a maladaptive response not previously documented in variant CGD. Neutrophils from two affected, but asymptomatic, siblings in the larger kindred were very similar in their rates of O_2^- and H_2O_2 production, depolarization responses, and NADPH oxidase kinetics.

The findings in these patients also raise fundamental questions about the process of O_2^- generation in normal granulocytes. Their ability to produce some O_2^- despite an absence of detectable cytochrome b deviates from the direct relationship between cytochrome content and O_2^- release reported in classic CGD patients and carriers. Furthermore, the defect in membrane potential change in these patients is mild compared with that of respiratory burst activity. These deviations from the relationships previously observed between cytochrome b, membrane depolarization, and O_2^- generation may require changes in the inferences drawn from classic CGD studies regarding the mechanisms of normal respiratory burst function.

CASE REPORTS

Family J. The propositus, Ra.J., presented in 1984 at age 15 with respiratory failure resulting from severe *Aspergillus* pneumonia, probably related to environmental exposure to bark mulch during a summer job on a gardening crew (Fig 1, left panel) He recovered completely after a nearly 2-month hospitalization, with treatment including mechanical ventilation, tracheostomy, amphotericin B, and additional antibiotics (ampicillin and tobramycin) for nosocomial infections with *Pseudomonas aeruginosa* and *Strepto-*

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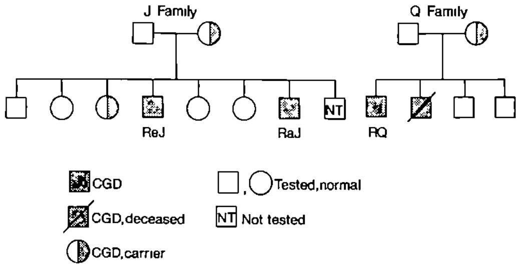

Fig 1. Pedigrees of families J. and Q. Disease status was determined by nitroblue tetrazolium slide testing as described in Materials and Methods.

coccus faecalis. His past medical history was unremarkable, with no serious or unusual infections Evaluation of other available family members by nitroblue tetrazolium slide tests revealed CGD in one brother (Re J.), still entirely asymptomatic at age 9. The mother and one sister proved to be carriers, with slide tests showing mixed populations of normal and deficient cells, the latter identical in appearance to those of the affected brothers (see Results).

Family Q. R Q was found to have CGD when his granulocytes were tested for nitroblue tetrazolium reduction shortly after his birth in 1977 (Fig 1, right panel). His brother, previously reported by Orson and Greco,[20] had died at the age of 2 months of a disseminated *Torulopsis* species infection, with the diagnosis of CGD performed by nitroblue tetrazolium reduction testing during the terminal illness. R Q. had been hospitalized twice for perirectal abscesses (first developed at the age of 18 months) and twice for pneumonia. All infections have responded to prolonged treatment with broad-spectrum antibiotics, including combinations of semisynthetic penicillins (primarily nafcilin and dicloxacillin), aminoglycosides, and trimethoprim/sulfamethoxazole.

MATERIALS AND METHODS

Phorbol myristate acetate (PMA) was purchased from Consolidated Midland Corp, Brewster, NY; Ficoll 400, dextran 500, and Percoll from Pharmacia Fine Chemicals, Piscataway, NJ; sodium Hypaque, 50%, from Sterling Winthrop, New York; arachidonic acid from Nu-Check Prep, Elysian, Minn; rabbit antibovine serum albumin from Cappel Laboratories, West Chester, Pa; quin-2 acetoxymethyl ester, from Calbiochem-Behring Corp, Los Angeles, and 3,3′-dipropylthiodicarbocyanine [di-S-C_3(5)], from Molecular Probes, Junction City, Ore. All other materials were obtained from Sigma Chemical Co, St Louis. All reagents were obtained in the purest form available and used without further purification unless otherwise indicated.

Peripheral venous blood in citrate anticoagulant was obtained from CGD patients and from normal volunteer donors and the neutrophils isolated by dextran sedimentation and Ficoll-Hypaque centrifugation [21] Procedures and consent forms were approved by the University of Massachusetts Medical Center and The Children's Hospital (Boston) Committees on the Protection of Human Subjects in Research

Superoxide production from pooled cells was measured by a previously described[21-23] continuous spectrophotometric assay of superoxide dismutase–inhibitable cytochrome c reduction Stimuli were PMA (1 μg/mL), arachidonic acid (0.082 mol/L, prepared as in reference 24), and antigen-antibody complexes (prepared from bovine serum albumin and rabbit antibovine serum albumin as described by Ward and Zvaifler[25] and used at a saturating dose of 240 μg/mL) Single-cell analysis for PMA-stimulated nitroblue tetrazolium reduction was performed by nitroblue tetrazolium slide tests as previously described[26] using either purified neutrophil suspensions or whole-blood clots as cell sources, with equivalent results.

H_2O_2 production was assayed by a modification[27] of the ferrithiocyanate method of Thurman et al [28]

The membrane potential change in response to PMA and antigen-antibody complexes (concentrations and preparation as described for the O_2^- assay) was measured as the change in emission fluorescence of the dye di-S-C_3^5 as previously described.[8] The initial linear rate of change (ΔF/min) and the extent of depolarization (ΔF) of the experimental subject's cells (a measure of the rate of depolarization) were compared with those of a concurrent normal control The data are expressed as a percentage of normal for the rate of change; virtually identical results were obtained for the extent of depolarization as well, but the data are not presented because of the possible artifact introduced by the differing rates of probe oxidation by the CGD and normal cells.[9]

NADPH oxidase activity was measured as NADPH-dependent superoxide dismutase–inhibitable cytochrome c reduction at 28 °C in particulate fractions from PMA-stimulated neutrophils.[29,30] The NADPH concentration was varied from 0.01 to 4 mmol/L and the data analyzed by the method of Lineweaver and Burk [31]

Cell disruption by nitrogen cavitation and fractionation on discontinuous Percoll gradients was performed as previously described.[32,33] The resultant three visible bands (α, β, and γ) were separately aspirated, the Percoll removed, and the band contents assayed for cytochrome b, flavoprotein, myeloperoxidase, and vitamin B_{12}–binding protein by previously reported methods [32-36] Cytochrome b content was also measured in whole cells by dithionite difference spectroscopy of cell suspensions in phosphate-buffered saline with 0.2% Triton X-100 [34]

Intracellular calcium flux was assessed with quin-2 fluorescence by a modification of the method of Lew et al.[37] Normal and patient cells (4×10^7) were preincubated in 1 mL of NaCl, 150 mmol/L; KCl, 5 mmol/L; HEPES, 10 mmol/L; $CaCl_2$, 1.3 mmol/L; and $MgCl_2$, 1 3 mmol/L (ph 7 4) containing 50 μmol/L quin-2 acetoxymethyl ester for 60 minutes at 37 °C. Cells were then washed and resuspended at 5×10^6/mL in the same buffer containing Ca^{++} (1 mmol/L) or in calcium-free buffer with 1 mmol/L EDTA. The neutrophils were preincubated in a thermostatted Perkin-Elmer Model 650-10S fluorometer (Perkin-Elmer Corp, Norwalk, Conn) at 37 °C for two minutes and then stimulated from either formylmethionylleucylphenylalanine (10^{-7} mmol/L), PMA (150 ng/mL), or arachidonic acid (100 μmol/L) and compared with untreated control cells. The time course of fluorescence change was measured in arbitrary fluroescence units at an excitation wavelength of 339 nm and an emission of 429 nm.

RESULTS

The respiratory burst activities of the variant CGD patients are presented in Table 1. The brothers Re.J. and Ra.J. produced similar amounts of O_2^- and H_2O_2 in response to a variety of stimuli acting at different stages of the activation process. Their cells' activity varied from 9% to 30% of normal, depending upon the stimulus. R.Q. produced O_2^- at a very low rate, 0.5% that of normal controls and well below that of other reported variant CGD patients. Neither patient nor control granulocytes produced any detectable superoxide at rest (data not shown).

During severe fungal or bacterial infection (*Aspergillus* pneumonia in Ra.J. and perirectal abscess in R.Q.), activity fell dramatically. In Ra.J., granulocytes collected during illness showed 10% to 36% the O_2^--generating rate and 20% the H_2O_2-generating rate of those collected after recovery.

Table 1. Respiratory Burst Function by Variant CGD Granulocytes

Function	Stimulus	Re.J.	Ra J	Ra.J *	R Q.	R Q.*	Normal
O_2^- production							
Rate (nmol/min/10^6 cells)	PMA	0.79	0.74	0.27	0.28	046	8.19 ± 0.21
	Arachidonate	1 17	1.03	<0.1	0.22	ND	4.94 ± 0.87
	Ag/Ab	0 38	0.75	0 75	ND	ND	3.34 ± 0.86
Lag time (s)	PMA	69	74	155	67	144	70 ± 4
H_2O_2 production							
Rate (nmol/min/10^6 cells)	PMA	1.61	1.43	0 28	ND	ND	6.35 ± 0 35

Abbreviations· ND, not determined; Ag/Ab, antigen-antibody complexes.

*Measurements performed during severe infection (see Case Reports).

Data for the patients represent the means of three to six experiments, with ranges less than 20% of the means, and data for normal controls represent the mean ± SD of ten experiments (O_2^-) and five experiments (H_2O_2) Unpaired *t* tests showed $P <$ 01 for comparisons to normal values for all patient O_2^- and H_2O_2 rates and for Ra.J.* and R.Q * lag times.

R.Q.'s granulocytes fell to undetectable levels of O_2^- production during bacterial infection (perirectal abscess). Nitroblue tetrazolium slide tests, performed at the same time as the pooled cell O_2^- measurements, showed the respiratory burst response of the CGD cells to be homogeneously distributed; that is, each cell contained a few grains of formazan rather than the heavy deposits seen in normal neutrophils. When the total O_2^- production was diminished during infection, the homogeneous pattern persisted, indicating a decreased response in all cells rather than a fall in the proportion of responding cells.

The lag time, a measure of the time required for activation of NADPH oxidase following stimulation,[24,25] was normal (58 to 72 seconds) in PMA-stimulated granulocytes from all three variant CGD patients but lengthened to twice normal during periods of infection.

Peripheral blood white cell counts (WBC) and differential counts on Ra.J. during infection and in health showed, respectively, 17 to 29,000 WBC/μL with 75% to 90% polymorphonuclear and 5% to 7% band forms and 8 to 13,000 WBC/μL with 50% to 65% polymorphonuclear and no band forms. WBC and differential counts on R.Q. during infection and in health showed, respectively, WBC 15 to 25,000 WBC/μL with 55% to 62% polymorphonuclear and 0% to 2% band forms and 8 to 16,000 WBC/μL with 27% to 46% polymorphonuclear and 0% to 2% band forms.

Assays of O_2^- production by normal granulocytes were also performed in the presence of 10% patient's serum or of patient's granulocytes in a 1:1 ratio to normal cells. At times of infection, when Ra.J and R.Q. showed decreased respiratory burst activity, neither their serum nor their cells affected the rate or lag time for O_2^- by normal cells. Measurements of O_2^- production and membrane potential change performed on granulocytes from Ra.J. and Re.J. during chicken pox infection showed no change from those obtained when they were healthy.

The membrane depolarization response, one of the earliest events in activation,[8,9] is presented in Table 2. The rate of change in membrane potential in response to PMA was diminished in all three CGD patients' granulocytes and fell further during infection in Ra.J. The responses to antigen-antibody complexes were normal in the J. brothers when well but dropped dramatically during infection. R.Q.'s granulocytes showed a diminished response even when he was healthy. All patients' granulocytes showed normal resting membrane potentials prior to stimulation, indicating that they had not been previously activated.

The rise in intracellular calcium, another early component of granulocyte activation,[37] was normal in Ra.J.'s cells stimulated with formylmethionylleucylphenylalanine, PMA, or arachidonate at the time of infection (data not shown) when O_2^- production and membrane depolarization were decreased. The assay was calibrated only for the change in calcium concentration, not its absolute value, so the resting calcium level could not be determined.

To further define the defect in cellular O_2^- production, we examined the kinetics of NADPH-dependent O_2^- generation in a particulate fraction prepared from granulocytes by sonication and differential centrifugation. As shown in Table 3, all three variant CGD patients had NADPH oxidase with both altered substrate affinity (high K_m^{app}) and decreased maximal velocity. Enzyme preparations from Ra.J.'s granulocytes during health and illness showed no appreciable difference, in contrast to the major changes in whole-cell O_2^- generation (Table 1). In mixing experiments, combinations of particulate fractions from Ra.J.'s and normal granulocytes showed no significant inhibition of the normal NADPH oxidase activity.

Cytochrome b, a component of the NADPH oxidase that is measurable independently from its enzymatic activity, was undetectable in all three patients' granulcotyes (Table 4). However, analysis of subcellular fractions of Ra.J.'s granulocytes showed a normal distribution of other markers including myeloperoxidase, predominantly in the α-band (primary

Table 2. Rate of Membrane Depolarization by Variant CGD Granulocytes

Stimulus	Re.J	Ra.J	Ra.J *	R.Q
PMA	38	41	16	34
Ag-Ab	91	113	4.6	22

*Measurements performed during severe infection (see Case Reports).

Data represent means of two experiments, each performed in duplicate or triplicate, with less than 20% difference between experiments. Values are the percentages of concurrent normal controls.

Table 3. Michaelis Constant and Maximal Velocity of NADPH Oxidase from Variant CGD Granulocytes

	Re.J.	Ra.J	Ra J *	R Q.	Normal
Michaelis constant (K_m^{app}) (mmol/L)	0.77	1 44	1.03	8 40	0.074
Maximal velocity (V_{max}) (nmol O_2^-/min/mg protein)	3.70	3.06	2.25	2.23	32 4

*Measurements performed during acute infections (see Case Reports).

Results represent the result of Lineweaver-Burk[31] calculations performed on pooled data from three (patient) and eight (control) experiments.

granule); and vitamin B_{12}–binding protein, in the β-band. A flavin adenine dinucleotide–containing flavoprotein, another constituent of NADPH oxidase reported to be absent in some CGD patients' granulocytes,[14,33] was present in normal quantity and subcellular distribution (Table 4) including the γ-fraction, the site of NADPH oxidase activity.

DISCUSSION

The present studies document the cellular and biochemical processes involved in granulocyte O_2^- production in three patients from two kindreds with variant CGD. All three show decreased, but detectable, O_2^- generation by abnormal NADPH oxidases that have both diminished substrate affinity for NADPH (high K_m^{app}) and decreased maximal velocities of O_2^- production. Their granulocytes also have abnormal rates of stimulus-induced membrane depolarization and undetectable cytochrome b.

A striking finding in two patients was the threefold to tenfold decrease in O_2^- production accompanying serious infection in Ra.J. (*Aspergillus* pneumonia) and R.Q. (perirectal abscess). The prolongation of the lag time and decrease in the rate of membrane depolarization during infection along with the lack of change in the kinetics of activated NADPH oxidase suggest an acquired defect in cell activation rather than a direct effect on the O_2^--generating enzyme. Transient abnormalities in bactericidal activity have been reported in otherwise normal patients at times of infection, but specific examination of respiratory burst function has shown an increase in activity in most studies and a decrease in others (reviewed in reference 38). This variability may represent the end product of the opposing effects of neutrophil priming[39] *v* deactivation,[40] exhaustion,[41] immaturity,[42] and effects of antibiotics.[43] Thus the change observed in variant CGD cells may represent an exaggeration of the normal granulocyte response to infection or a unique problem related to the described total activation defect in classic CGD.[8,9] The residual O_2^--generating activity of CGD variants helps to explain their relative freedom from the recurrent infections of the classic disease. However, the marked decrease described in the present study indicates the potential for a vicious cycle in which an infection, once established, leads to increasing impairment of host defense.

The two brothers, Ra J. and Re.J., show very similar granulocyte O_2^- production and NADPH oxidase kinetics. Thus, there is no evidence of variability in expression of the variant CGD genetic defect in this family, at least in times of health. In contrast, the patients described by Lew et al[15] and by Styrt and Klempner,[18] although second cousins, showed fivefold differences in PMA-stimulated O_2^- production and in the maximal velocity for particulate fraction NADPH oxidase activity. However, the assays were performed by different laboratories at different times.

The absence of cytochrome b from the granulocytes of these patients with X-linked variant CGD is consistent with the genetic pattern observed in classic CGD[13] and in two previously reported variant cases[16] but not in a third apparent X-linked variant.[19] If the cytochrome b content were proportional to the residual rate of O_2^- production in CGD variants, it should have been detectable in the J. family. Its absence suggests the possibility of a cytochrome-independent alternative pathway of O_2^- production in variant CGD granulocytes; the kinetic data indicate that the pathway might have less affinity for NADPH and lower reaction velocity than the normal oxidase. Alternatively, an amount of cytochrome b below the limit of detection may suffice for the O_2^- production observed. In resting granulocytes, most of the cytochrome is found in the secondary granules and only 12% in

Table 4. Cytochrome b Content of Granulocytes and Distribution of Markers in Subcellular Fractions

Subject	Fraction*	Cytochrome b (pmol/mg Protein)	Flavoprotein (pmol/mg Protein)	Myeloperoxidase (U/mg protein)	Vitamin B_{12}–Binding Protein (ng/mg Protein)
Re.J.	whole cells	<10			
R Q	whole cells	<10			
Normal	whole cells	101 ± 58			
Ra J.	α	<10	0	4.3	0 02
	β	<10	82	2 7	18 0
	γ	<10	60	0.4	0.59
	S_2	<10	12	0	0 03
Normal	α	<10	0	7 9	0 12
	β	442	147	2 3	16.7
	γ	225	83	0	0.39
	S_2	<10	15	0	0.29

*Bands from discontinuous Percoll gradients as described in Materials and Methods and defined in reference 32

Results for whole-cell cytochrome b represent data from four experiments (patients) and the mean ± SD for four experiments (normal). Data for cell fractions are presented from one experiment, with results (except for the absence of cytochrome b in RaJ cells) similar to those previously reported from the same laboratory.[32-34]

the plasma membrane.[32,33] Possibly, only the latter, the minority, of the total cell content might be essential for O_2^- respiratory burst activity. In contrast, these and some other CGD patients present a different problem for the theoretic connection between plasma membrane potential change and O_2^- production. In classic CGD granulocytes, there is no membrane potential response to phagocytic stimuli,[8,9] whereas in the variant CGD granulocytes, the depolarization response is normal or, if decreased, not nearly in proportion to the defect in O_2^- generation. That is, the membrane depolarization and respiratory burst defects do not remain proportional in CGD variants, providing an example of a dissociation between the two processes and indicating that normal depolarization is not sufficient to initiate a normal respiratory burst. Thus these cases, like other rare variants of unusual diseases, represent experiments of nature that can guide experiments in the laboratory to the elucidation of normal as well as pathologic physiology.

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REFERENCES

1 Donowitz GR, Mandell GL. Clinical presentations and unusual infections in chronic granulomatous disease, in Gallin JI, Fauci AS (eds): Chronic Granulomatous Disease. New York, Raven, 1983, p 55

2. Tauber AI, Borregaard N, Simons ER, Wright J: Chronic granulomatous disease. A syndrome of phagocyte oxidase deficiencies. Medicine (Baltimore) 62:286, 1983

3 Curnutte JT, Whitten DM, Babior BM: Defective superoxide production by granulocytes from patients with chronic granulomatous disease. N Engl J Med 290:593, 1974

4. Babior BM: The nature of NADPH oxidase, in Gallin JI, Fauci AS (eds)· Chronic Granulomatous Disease. New York, Raven, 1983, p 91

5 Badwey JA, Karnovsky ML· Active oxygen species and the function of phagocytic leukocytes Annu Rev Biochem 49·695, 1980

6. Hohn DC, Lehrer RI: NADPH oxidase deficiency in X-linked chronic granulomatous disease. J Clin Invest 45:1422, 1967

7 McPhail LC, DeChatelet LR, Shirley PS, Wilfert C, Johnston RB Jr, McCall CE· Deficiency of NADPH oxidase activity in chronic granulomatous disease. J Pediatr 90:213, 1977

8. Whitin JC, Chapman CE, Simons ER, Chovaniec ME, Cohen HJ: Correlation between membrane potential changes and superoxide production in human granulocytes stimulated with phorbol myristate acetate. J Biol Chem 255 1874, 1980

9. Seligman BE, Gallin JI: Use of lipophilic probes of membrane to assess human neutrophil activation. J Clin Invest 66:493, 1980

10. Mills EL, Quie PG: Inheritance of chronic granulomatous disease, in Gallin JI, Fauci AS (eds): Chronic Granulomatous Disease. New York, Raven, 1983, p 25

11 Shinagawa Y, Tanaua C, Teraoka A, Shinagawa Y· A new cytochrome in neutrophilic granules of rabbit leukocytes. J Biochem 59 622, 1966

12. Segal AW, Jones OTG, Webster D, Allison AC: Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. Lancet 2:446, 1978

13. Segal AW, Cross AR, Garcia RC, Borregaard N, Valerius NH, Foothill JF, Jones OTG· Absence of cytochrome b_{-245} in chronic granulomatous disease· A multicenter European evaluation of its incidence and relevance N Engl J Med 308:245, 1983

14. Gabig TG. The NADPH-dependent O_2^--generating oxidase from human neutrophils: Identification of a flavoprotein component that is deficient in a patient with chronic granulomatous disease. J Biol Chem 258:6352, 1983

15 Lew PD, Southwick FA, Stossel TP, Whitin JC, Simons E, Cohen HJ. A variant of chronic granulomatous disease: Deficient oxidative metabolism due to a low-affinity NADPH oxidase. N Engl J Med 305:1329, 1981

16. Seger RA, Tiefenauer L, Matsunaga T, Wildfeuer A, Newburger PE: Chronic granulomatous disease due to granulocytes with NADPH oxidase activity and deficient cytochrome b Blood 61:423, 1983

17. Shurin SB, Cohen HJ, Whitin JC, Newburger PE· Impaired granulocyte superoxide production and prolongation of the respiratory burst due to a low-affinity NADPH-dependent oxidase. Blood 62:564, 1983

18 Styrt B, Klempner MS: Late-preventing variant of chronic granulomatous disease Pediatr Infect Dis 3·556, 1984

19 Borregaard N, Cross AR, Herlin T, Jones OTG, Segal AW, Valerius NH A variant form of X-linked chronic granulomatous disease with normal nitroblue tetrazolium slide test and cytochrome b. Eur J Clin Invest 13.243, 1983

20. Orson JN, Greco RG: Death from *Torulopsis* in chronic granulomatous disease. J Pediatr 87:333, 1975 (letter)

21 Babior BM, Cohen HJ: Measurement of neutrophil function· Phagocytosis, degranulation, the respiratory burst, and bacterial killing, in Cline MJ (ed)· Leukocyte Function. New York, Churchill Livingstone, 1981, p 1

22. Cohen HJ, Chovaniec ME. Superoxide production by guinea pig granulocytes A basis for a continuous assay monitoring superoxide production and for the study of the activation of the generating system. J Clin Invest 61.1081, 1978

23. Newburger PE, Chovaniec ME, Cohen HJ: Activity and activation of the granulocyte superoxide generating system Blood 55·85, 1980

24. Badwey JA, Curnutte JT, Karnovsky ML. *cis*-Polyunsaturated fatty acids induce high levels of superoxide production by human neutrophils. J Biol Chem 256:12640, 1981

25 Ward PA, Zvaifler NJ. Quantitative phagocytosis by neutrophils: I. A new method with immune complexes. J Immunol 111 1771, 1973

26. Newburger PE, Cohen HJ, Rothchild SB, Hobbins JC, Malawista SE, Mahoney MJ· Prenatal diagnosis of chronic granulomatous disease. N Engl J Med 300.178, 1979

27. Speier C, Baker SS, Newburger PE: Relationships between in vitro selenium supply, glutathione peroxidase activity, and phagocytic function in the HL-60 human myeloid cell line. J Biol Chem 260.8951, 1985

28. Thurmon RG, Ley HG, Scholz R· Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. Eur J Biochem 25·420, 1972

29. Suzuki Y, Lehrer RI: NADPH oxidase activity in human neutrophils simulated by phorbol myristate acetate. J Clin Invest 66:1409, 1980

30. Light DR, Walsh C, O'Callaghan AM, Goetzl EJ, Tauber AI· Characteristics of the cofactor requirements for the O_2^--generating NADPH-oxidase of human polymorphonuclear leukocytes. Biochemistry 20:1468, 1981

31. Lineweaver H, Burk D. The determination of enzyme dissociation constants. J Am Chem Soc 56.658, 1934

32. Borregaard N, Heiple JM, Simons ER, Clark RA: Subcellular localization of the b-cytochrome component of the human

neutrophil microbicidal oxidase: Translocations during activation J Cell Biol 97·52, 1983

33. Borregaard N, Tauber AI· Subcellular localization of a human neutrophil NADPH-oxidase: b-cytochrome and associated flavoprotein. J Biol Chem 259:47, 1984

34. Higson FK, Durbin L, Pavlotsky N, Tauber AI Studies of the cytochrome b_{-245} translocation in the PMA stimulation of the human neutrophil NADPH-oxidase. J Immunol 135:519, 1985

35. Kanofsky JR, Wright J, Miles-Richardson GE, Tauber AI: Biochemical requirements for singlet oxygen production by purified human myeloperoxidase. J Clin Invest 74.1489, 1984

36. Gottlieb C, Lau KS, Wasserman LR, Herbert V: Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity Blood 25:875, 1965

37. Lew PD, Wollheim CB, Waldvogel FA, Possan T: Modulation of cytosolic free calcium transients by changes in intracellular calcium buffering capacity Correlation with exocytosis and O_2^- production in human neutrophils. J Cell Biol 99:1212, 1984

38. Klebanoff SJ, Clark RA: The Neutrophil: Function and Clinical Disorders Amsterdam, North-Holland, 1978, p 597

39. English D, Roloff JS, Lukens JM: Chemotactic factor enhancement of superoxide release from fluoride and phorbol myristate acetate stimulated neutrophils Blood 58:129, 1981

40. Ward PA, Becker EL: The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. J Exp Med 127.693, 1968

41. Matzner Y, Brass LM, McMurrich BJ, Peters WA, André-Schwartz J, Babior BM· Expression of a chronic granulomatous diseaselike defect by fluoride-exhausted neutrophils Blood 60.822, 1982

42. Zakhireh B, Root RK: Development of oxidase activity of human bone marrow granulocytes. Blood 54:429, 1979

43. Duncker D, Ullman U: Influence of various antimicrobial agents on the chemiluminescence of phagocytosing human granulocytes. Chemotherapy 32:18, 1986

Ultrastructural Studies of the Interaction of Spectrin With Phosphatidylserine Liposomes

By Amos M. Cohen, Shih-Chun Liu, Laura H. Derick, and Jiri Palek

Spectrin was shown previously to interact with phosphatidylserine and phosphatidylethanolamine, which are preferentially localized in the inner half of the membrane lipid bilayer, but this interaction is not well characterized. In the present study we used electron microscopy of rotary-shadowed platinum replicas of spectrin dimer–phosphatidylserine complexes to study the interaction of spectrin with phosphatidylserine vesicles. At a spectrin concentration of 0.6 mg/mL, 60% of spectrin dimers were associated with phosphatidylserine vesicles and at a spectrin concentration of 1.2 mg/mL, some vesicles were crosslinked by spectrin dimers. The length of the protruding segment of spectrin dimer from the liposome edge ranged from 400 to 960A° and the contact region to phosphatidylserine extended 272 ± 144A° from either end of the molecule. Therefore, these data are consistent with multiple binding sites to phosphatidylserine throughout the spectrin dimer molecule. Spectrin tetramers, when bound to phosphatidylserine liposomes, extended 1804 ± 79A° from the liposome edge and crosslinked liposomes, suggesting that some of the binding sites to phosphatidylserine vesicles is in the proximity of the tail end of spectrin. The association between spectrin dimers to phosphatidylserine was demonstrated by nondenaturing gel electrophoresis. The complexes were separated into multiple bands with molecular weight of 1.4×10^6, 1.8×10^6, and 2.3×10^6. These bands did not represent self-associated spectrin oligomers, since postincubation treatment with Triton-X-100 dissociated them into spectrin dimers. Furthermore, these spectrin high molecular weight bands, as visualized by Coomassie blue absorbance, closely corresponded to the ^{14}C-phosphatidylserine distribution. These data provide ultrastructural and biochemical evidence that spectrin binds to phosphatidylserine at multiple sites including the tail end region.
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SPECTRIN, the major protein of the erythrocyte membrane skeleton, is composed of two nonidentical subunits (α chain, 240,000 daltons, and β chain, 220,000 daltons) that are associated into double-stranded fiberlike flexible heterodimers about 1000°A in length.[1] Recently, amino acid sequencing studies suggested that spectrin is composed of multiple triple helical segments, with a 106 amino acid repeating subsegment that seemed to extend throughout the length of both subunits.[2] Spectrin heterodimers (SpD) are assembled head to head into tetramers (SpT) and higher order oligomers (SpO).[3,4] These species are interconnected in a continuous submembrane network by their interaction with oligomeric actin, an association that is modulated by protein 4.1.[5–10] The submembrane spectrin-actin network is attached to the asymmetric lipid bilayer through ankyrin (band 2.1) and protein 4.1, which, in turn, interact with the transmembrane proteins, band 3 and glycophorins A and C.[11–13]

While the protein interactions involved in attachment of the skeleton to the membrane are relatively well characterized, evidence for the interaction between spectrin and the lipids of the inner bilayer is mainly indirect. Spectrin has been shown to interact with aminophospholipids in studies employing lipid monolayers,[14] phospholipid vesicles,[15] and intact human erythrocytes (RBC).[16] Treatment of RBC by sulfhydryl-oxidizing agents (diamide and tetrathionate) causes both phosphatidylserine (PS) and phosphatidylethanolanine (PE), which are located mainly in the inner half of the lipid bilayer, to be hydrolyzed by extracellular phospholipase A_2, suggesting a reorientation of the lipids to the outer half of the lipid bilayer.[16,17] Likewise, diamide increased the transbilayer reorientation rate of incorporated lysophospholipids [18] However, heat denaturation of spectrin in RBC did not alter lysophosphatidylcholine translocation rate in the lipid bilayer.[19] Spectrin-deficient RBC of a mouse variant strain with spherocytosis were found to have partial redistribution of membrane phospholipids, as shown by substantial increase of PE in the outer lipid bilayer leaflet with a concomitant translocation of phosphatidylcholine (PC) from the outer to the inner bilayer leaflet.[20] Recently, studies of RBC phospholipid distribution in a patient with severe hereditary spherocytosis and decreased spectrin content also revealed redistribution of phospholipids as shown by significant increase in the content of outer lipid leaflet PS and PE and decrease in PC.[21] The fluorescent probe merocyanine 540, which binds preferentially to lipid bilayers that are in a fluid state, was found to selectively stain erythrocyte membranes in several systems in which the underlying spectrin network was altered or missing, further suggesting that spectrin interacts with the membrane lipid bilayer.[22] Differential scanning calorimetry studies of dimyristoyl-PS vesicles showed a decrease in enthalpy change of the lipid phase transition upon addition of spectrin-actin extract.[15] These vesicles collapsed and fused into multilamellar structures in the presence of spectrin-actin as demonstrated by freeze etching electron microscopy and ^{31}P nuclear magnetic resonance.[15] High spectrin penetration into a lipid monolayer, as recorded by a surface area increase, was observed with PS while PC, a predominant constituent of the outer lipid bilayer, showed only poor spectrin penetration.[14] In addition, spectrin was shown to have a large number of binding sites for hydrocarbon chains as revealed by hydrophobic ligands that quenched intrinsic protein fluorescence.[23] Recent studies of anilinonaphtyl-labeled spectrin revealed that spectrin exhibits a spectrum characteristic of a highly hydrophobic environment and it binds PS vesicles at a ratio of one

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tetramer to 750 exposed phospholipids with an affinity of about 3×10^7 mol/L^{-1}.[24]

In this communication, we provide direct ultrastructural and biochemical evidence that spectrin binds to PS liposomes at multiple sites including the tail end region.

MATERIALS AND METHODS

Materials. ^{14}C-phosphatidylserine, 1,2-dioleoyl, 60 m Ci/mmol, was purchased from Amersham. Protosol, toluene, and Liquelfluor were obtained from New England Nuclear. Bovine brain phosphatidylserine (PS) was purchased from Calbiochem, Avanti, and Pharmacia-PL Biochemicals and were 99% pure by TLC. All other chemicals were obtained from Sigma unless otherwise specified.

Spectrin extraction. Freshly drawn blood was filtered through cellulose and washed to remove white cells. Ghosts were prepared from washed erythrocytes by hypotonic lysis by the method of Dodge et al.[26] Ghosts in 3 mmol/L Na_3PO_4 (pH 8.0) were treated with 0.5 mmol/L diisopropyl fluorophosphate (DFP) at 0 °C for ten minutes to inactivate endogenous proteases. For low ionic strength extraction of spectrin, DFP-treated ghosts were washed with 0.1 mmol/L Na_3PO_4 (pH 8.0) and immediately centrifuged to reduce the ionic strength of the medium and obtain a smaller pellet. Ghosts were then incubated at 37 °C for 30 minutes to obtain SpD-enriched extract (or at 0 °C for 16 hours for the study of SpT), with an equal volume of low ionic strength buffer containing 0.1 mmol/L Na_3PO_4, 0.1 mmol/L EDTA, 0.1 mmol/L dithiothreitol (DTT), and 0.1 mmol/L Na N_3, pH 8.0. After incubation, the supernatant containing the crude spectrin extract and ghosts were separated by centrifugation at 250,000 × g, 4 °C, for 35 minutes. Crude spectrin extract was layered onto linear sucrose gradients (5% to 20% w/v) and centrifuged at 200,000 × g for 16 hours at 4 °C to obtain SpD or SpT fractions free of band 4.1. Protein concentration in collected fractions was determined by absorbance at 280 nm. To remove sucrose, the major peak fraction was dialyzed against 0.15 mol/L NaCl, 5 mmol/L TES, 1 mmol/L DTT, 1 mmol/L EDTA, and 0.15 mmol/L NaN_3 (pH 7.1) for 18 hours. Spectrin purity was tested by 5.6% acrylamide SDS gel electrophoresis. Spectrin dimers and SpT purity were tested by 2.5% acrylamide, 0.3% agarose (without SDS) nondenaturing gel electrophoresis.

Preparation of ^{14}C-phosphatidylserine liposomes. Purified bovine brain phosphatidylserine (PS) was used for electron microscopic studies or was mixed (9:1) with ^{14}C-phosphatidylserine 1.2 dioleoyl for electrophoretic studies and sonicated (Heat System Ultrasonics, Plainview, NY, model 185 F, 200 watt) for 30 minutes at 0 °C under N_2 to prevent oxidation. Under these conditions, small unilamellar vesicles are formed.[27] Metal debris and undispersed lipid were removed by centrifugation at 200,000 × g for 30 minutes at 4 °C. PS concentration was determined by phosphate analysis by the method of Ames.[28] The liposomes were kept under N_2 atmosphere at 0 °C, and used within four hours after preparation to prevent oxidation and aggregation.

Ultrastructural analysis of spectrin-PS complexes by low angle rotary shadowing. Spectrin dimers or spectrin tetramers (0.6 to 1.2 mg/mL) were incubated with PS vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C (a temperature at which spectrin dimer-tetramer transformation is kinetically "frozen") for 120 minutes. After incubation, aliquots were added directly to glycerol at 0 °C to yield a glycerol concentration of 60%. Samples of spectrin or spectrin-PS complexes were sprayed onto freshly cleaved mica, dried under vacuum, and shadowed on a rotating stage with platinum-carbon from an electron bombardment gun (Balzers BAF 400D) at a shadow angle of 6° as described.[29] Replicas were coated with a support film of carbon, floated onto distilled water, mounted on 400-mesh copper grids, and examined with a JEOL JEM 100S electron microscope at 60 kV. Samples of pure PS vesicles in 60% glycerol were sprayed onto freshly cleaved mica which was previously prepared by immersion into boiling 0.1% Alician blue 8GX for two minutes, rinsed with distilled water, and air dried, to promote lipid vesicle adhesion to the mica.[30] The sprayed PS vesicles were dried and shadowed in the same way as pure spectrin or spectrin-PS complexes.[29] Pure PS vesicle replicas were floated off the mica onto 5% sodium hypochloride, rinsed with distilled water, cleaned with chromic acid followed by distilled water rinses, and mounted on 400-mesh copper grids and studied in the same way as pure spectrin or spectrin-PS complexes. Assessment of spectrin binding sites to PS vesicles was made by examination of SpD-PS and SpT-PS complexes at ×125,000 by 0.1 mm graduated X7 measuring magnifier (Bausch and Lomb). The distance between SpD or SpT end and PS vesicle edge was measured and subtracted from SpD or SpT native length (1000°A and 2000°A, respectively),[1] which yielded spectrin binding region length and an unbound segment of spectrin.

Electrophoresis of spectrin-PS complexes. Quantitation of protein-lipid binding by direct methods requires the separation of the unbound protein from the protein-lipid complexes. A previous method that employed sedimentation of dextran loaded vesicles to quantitate spectrin-lipid binding[39] was found in our hands to show low recovery of the SpD-PS complexes, due to leakiness of the PS vesicles (unpublished data). To evaluate SpD-PS binding we subjected SpD-PS mixture to nondenaturing gel electrophoresis to separate free SpD and SpD bound to PS. Purified SpD (0.1 mg/mL) was incubated with 1.0 mg/mL ^{14}C-PS liposomes at isotonic conditions 5 mmol/L TES, pH 7.1, 0.15 mol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L DTT, and 0.15 mmol/L Na N_3 at 0 °C for 120 minutes. To separate SpD-PS species by nondenaturing gel electrophoresis, samples that contained 10 ug SpD were mixed with one-ninth volume of concentrated buffer to give a final solution of 40 mmol/L Tris-HCl, pH 7.4, 20 mmol/L Na acetate, 2 mmol/L DTT, 200 mmol/L sucrose, and 2 mmol/L EDTA, and electrophoresed in a 0.3% agarose to 2.5% acrylamide gels (without SDS) at 20 volts for 22 hours as previously described.[31] Triton-X-100, a nonionic detergent, was used to dissociate spectrin species from PS vesicles for evaluation of SpD self-association into SpT and SpO. Control SpD-PS mixture, SpD or SpO were further incubated with 0.1% Triton-X-100 for ten minutes at 0 °C and electrophoresed under the same conditions. For tracing spectrin, gels were stained with Coomassie blue, frozen, and sliced into 1 mm sections. Each slice was then extracted by pyridine (25% v/v) and the absorbance at 605 nm was read for each slice. For counting ^{14}C-PS duplicate gels were frozen immediately and sliced into 1 mm sections. Each slice was then soaked in 5 mL scintillation fluid (toluene, Protosol, Liquelfluor) and counted for ^{14}C (Beckman, gamma 4000, Irvine, Calif) as described.[32]

RESULTS

Ultrastructure of spectrin dimer–phosphatidylserine complexes. As shown on Fig 1A and 1B, the length (1000°A for dimer) and thickness (50 to 80°A) of the SpD molecules are consistent with the published values.[1] The diameter of the PS liposomes (less than 500°A) as measured by low angle rotary shadowing was consistent with measurements of the liposomes by negative staining (data not shown) and with published values for the sonicated small unilamellar vesicles.[27] Spectrin dimer–PS complexes are shown in Fig 1A and 1B. In order to determine whether the SpD-PS complexes are genuine or artifact, we examined by low angle

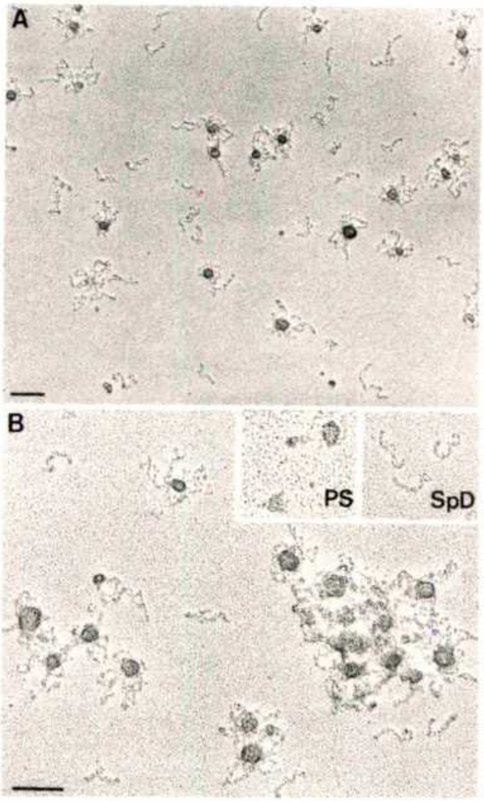

Fig 1. Electron micrography of low angle rotary shadowed platinum-carbon replicas of spectrin dimers and phosphatidylserine vesicles. (A) Spectrin dimer-phosphatidylserine (PS) complexes (low magnification). Spectrin dimers (1.2 mg/mL) were incubated with PS (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C for two hours. The sample was diluted to 60 ug/mL spectrin in 60% glycerol and sprayed and shadowed as described in Methods (Bar = 1000A°). (B) Spectrin dimer-phosphatidylserine complexes (high magnification), spectrin dimers (SpD), and phosphatidylserine (PS) vesicles (Bar = 1000A°).

Table 1. Number of Spectrin Dimers Bound per Phosphatidylserine Vesicles

Number of Spectrin Dimer Molecules Bound per a PS Vesicle	Percentage of Total Complexes
1	6
2	8
3	26
4	23
5	20
6	12
7	5

Spectrin dimers (0.6 mg/mL) were incubated with phosphatidylserine vesicles in isotonic conditions (pH 7.1) at 0 °C for 120 minutes. Electron micrographs of low angle shadowed platinum-carbon replicas of spectrin-PS complexes were studied for the number of spectrin dimer molecules bound per vesicle.

rotary shadowing the ultrastructure of actin, ankyrin, albumin, and protein 4.1, which were incubated with PS vesicles. The ultrastructure results showed that actin, ankyrin, and albumin did not associate with PS vesicles (data not shown), therefore suggesting that SpD-PS complexes are genuine. In contrast, protein 4.1 associated with PS vesicles, which is the subject of another communication (Cohen AM, Liu SC, Palek J, manuscript in preparation). At an SpD concentration of 0.6 mg/mL, and a PS concentration of 0.3 mg/mL, 68% (out of 318 SpD molecules counted) were bound to PS vesicles. This SpD concentration of 0.6 mg/mL was the highest at which no crosslinking of PS vesicles by SpD took place. The number of SpD molecules bound per PS vesicle was analyzed in 61 SpD-PS complexes and ranged between 1 and 7 SpD molecules bound per PS liposome. As shown in Table 1, complexes of SpD-PS that contained 3 SpD were most prevalent (26%). However, quantitation of the number of SpD molecules bound per lipid vesicle provided only an approximate estimation because of a relatively limited number of SpD-PS complexes available for quantitation, as well as a possible variation of adhesion SpD-PS complexes to the mica surface and fragmentation of crosslinked complexes during sample spraying.[29] Most of the SpD molecules' contact point to PS consisted of less than half of the SpD molecule length (Fig 2A to 2C). This observation enabled a crude estimation of the location of the spectrin binding sites for PS vesicles which was made by subtraction of the distance of the SpD that extended beyond the liposome edge from the native SpD length. Analysis of 63 SpD molecules bound to PS vesicles revealed the length range of the protruding segment of SpD from the liposome edge to be 400 to 960A° (Fig 3). The contact region of SpD with PS liposomes was calculated to be 272 ± 144A° (1 SD) from

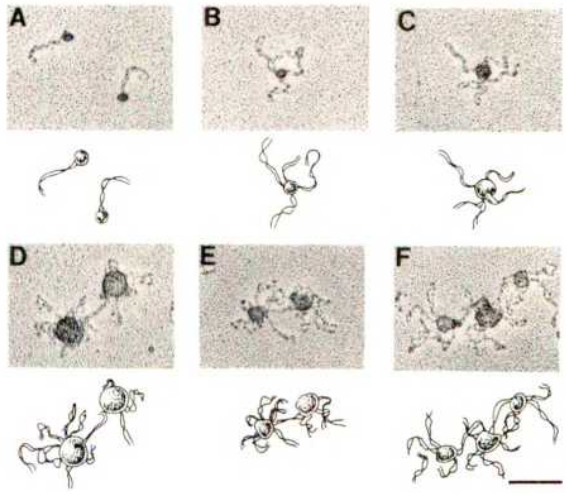

Fig 2. Electron micrographs of low angle rotary shadowed platinum-carbon replicas of spectrin dimer-phosphatidylserine (PS) complexes. Spectrin dimers (0.6 to 1.2 mg/mL) were incubated with PS vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C for two hours. Samples were then diluted in glycerol and treated as in Fig 1. (A) Two complexes of one spectrin dimer bound at one of its ends to PS vesicle. (B) A complex of 4 spectrin dimer molecules bound at one end to PS vesicle. (C) A complex of 5 spectrin dimer molecules bound to PS vesicle. (D,E) Two spectrin dimer–PS complexes crosslinked by one spectrin dimer molecule. The distance between the 2 PS vesicles is 720A° and 520A°, respectively. (F) Spectrin dimer–PS aggregate (Bar = 1000A°).

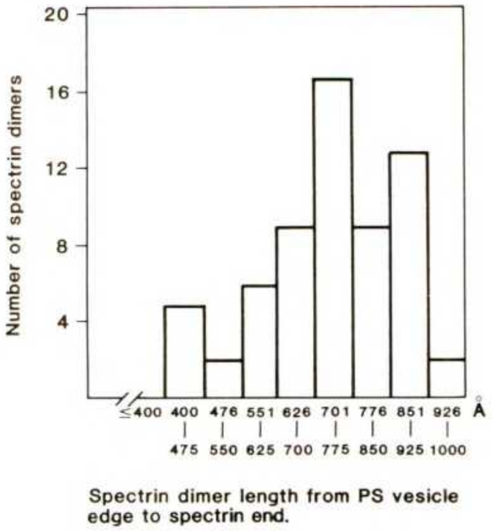

Fig 3. Histogram of spectrin dimer length from phosphatidylserine vesicle edge to spectrin end. Spectrin dimers (0.6 mg/mL) were incubated with phosphatidylserine vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C for two hours. Samples were then diluted in glycerol and treated as in Fig 1. The electron micrographs (×125,000) of 63 spectrin dimer molecules extending from liposome edge were measured by a ×7 measuring magnifier.

either end of the SpD molecule. These results appear to be considerably more variable than the site of spectrin-ankyrin interaction on β spectrin, which resides approximately 200°A from spectrin head end.[8,33] Thus, these data are consistent with the presence of multiple binding sites to PS throughout the SpD molecule length.

Crosslinking of phosphatidylserine vesicles by spectrin dimers. At spectrin concentration of 0.6 mg/mL, SpD decorated PS vesicles (Fig 2A to 2C) and at a concentration of 1.2 mg/mL, SpD produced considerable crosslinking of PS vesicles (Fig 2D, 2E). This suggests that both spectrin head and tail end regions bind to PS liposomes. Some SpD-PS complexes crosslinked by SpD had a distance of up to 720°A between bound vesicles (range 256 to 720°A) (Fig 2D, 2E). Furthermore, SpD crosslinked PS vesicles into small aggregates (Fig 1B and 2F), indicating that SpD molecule has several binding sites to PS.

Interaction of spectrin tetramers with phosphatidylserine. Electron micrographs of SpT-enriched preparations (with less than 10% SpD and SpO) and PS liposomes are shown in Fig 4A and 4B. The structure of SpT (2000°A length, 50 to 80A° thickness) was consistent with previously published values.[1] Spectrin tetramers were bound to PS vesicles mainly at one of their ends, constituting the SpD tail end (Fig 4C, Fig 5A to 5C). The average distance for 24 SpT molecules from liposome edge was 1804 ± 79°A (range 1760 to 1920°A), which suggested a binding site for PS located in a region within 196 ± 79°A from the spectrin tail end. Spectrin tetramers crosslinked PS vesicles with a distance of up to 1400°A between two bound vesicles (range 760 to 1400°A), which confirmed the involvement of several binding sites of SpT to PS (Fig 5D, 5E). In addition, incubation of SpT with PS vesicles leads to an extensive crosslinking of PS vesicles (Fig 5F). Both SpT and SpD, at the same mass concentration (1 mg/mL), crosslinked PS vesicles by a similar amount.

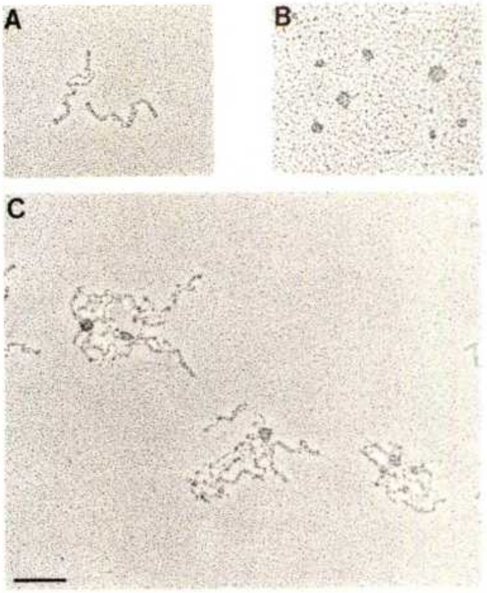

Fig 4. Electron micrograph of low angle rotary shadowed platinum-carbon replicas of spectrin tetramers and phosphatidylserine (PS) vesicles. Spectrin tetramers (0.6 mg/mL) were incubated with PS vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C for two hours. Samples were then diluted in glycerol and treated as in Fig 1. (A) Spectrin tetramers. (B) Phosphatidylserine vesicles. (C) Spectrin tetramer–phosphatidylserine complexes.

Separation of spectrin dimer–phosphatidylserine complexes by nondenaturing gel electrophoresis. In order to quantitate SpD binding to PS vesicles, we employed nondenaturing gel electrophoresis. We have previously used this method to separate the various size spectrin oligomers from membrane extracts.[31] Originally we employed large multilamellar PS vesicles, but these vesicles barely entered the gels and decreased the mobility of unbound SpD, thus preventing valid quantitation of binding. Small unilamellar PS vesicles moved freely through the gel and concentrated behind the dye front region and therefore were selected to study SpD-PS association. Complexes of SpD with ^{14}C-PS liposomes were separated by nondenaturing gel electrophoresis into multiple high molecular weight bands (Fig 6A). A short postincubation treatment with Triton-X-100 dissociated these bands predominantly into SpD, which exhibited a slightly faster mobility than the control SpD. This change in mobility was not a consequence of degradation of the spectrin, as confirmed by SDS gel electrophoresis of the SpD-PS complexes, dissociated by Triton-X-100, which showed that spectrin mobility was unchanged. Furthermore, spectrin oligomers were not dissociated by 1% Triton-X-100, as documented by nondenaturing electrophoresis (data not shown) which is consistent with a previous report.[34] Spectrin high molecular weight bands, as visualized by Coomassie blue absorbance, closely corresponded to the ^{14}C-PS distribution (Fig 6B). Thus, these 1.4×10^6, 1.8×10^6, 2.3×10^6,

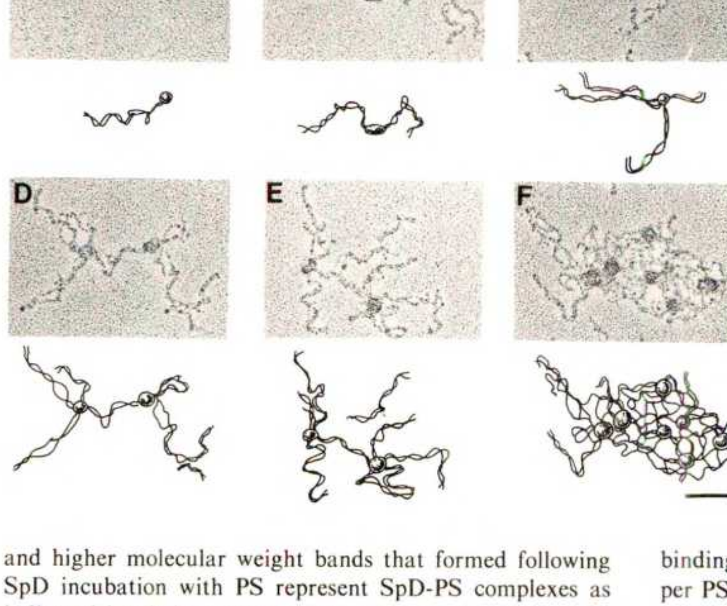

Fig 5. Electron micrograph of low angle rotary shadowed platinum-carbon replicas of spectrin tetramers and phosphatidylserine (PS) vesicles. Spectrin tetramers (0.6 mg/mL) were incubated with PS vesicles (0.3 mg/mL) in 5 mmol/L TES (pH 7.1), 0.15 NaCl, 1 mmol/L EDTA, and 1 mmol/L DTT at 0 °C for two hours. Samples were then diluted in glycerol and treated as in Fig 1. (A) A complex of one spectrin tetramer molecule bound at one end to PS vesicle. (B) A complex of two spectrin tetramer molecules bound at one end to PS vesicle. (C) A complex of three spectrin tetramer molecules and one spectrin dimer molecule bound to PS vesicle. (D,E) Two spectrin tetramer–PS complexes crosslinked by a spectrin tetramer molecule. The distance between the 2 PS vesicles is 1400A° and 1200A°, respectively. (F) Spectrin tetramer aggregate of 7 PS vesicles crosslinked into a quasi–two-dimensional structure.

and higher molecular weight bands that formed following SpD incubation with PS represent SpD-PS complexes as indicated by their complete disappearance upon treatment with Triton-X-100 and by the comigration of spectrin (Coomassie blue) and ^{14}C-PS in the gels. The spectrin to PS molar ratio varied throughout the high molecular bands region (0.8×10^{-3} to 6.5×10^{-3}) with a trend of higher molar ratios to coincide with the spectrin bands. This variability of the SpD to PS molar ratio probably reflected PS vesicles' mass heterogenicity and prevented quantitation of SpD to PS binding or estimation of the number of SpD molecules bound per PS vesicle. This pattern of high molecular band formation of SpD-PS complexes by nondenaturing gel electrophoresis was remarkably reproducible when three different sources of purified PS were tested, thus making the possibility of SpD interaction with a contaminant highly unlikely. We have also examined the SpD association with sphingomyelin, PC, and PE. Sphingomyelin and PC, the major constituents of the outer lipid bilayer, did not associate to a significant degree with SpD. In contrast, SpD formed similar high molecular weight complexes with the inner lipid bilayer constituents PE and a mixture of PE/PS (3:2 molar ratio). Both of these data are in agreement with previously published data.[14,39]

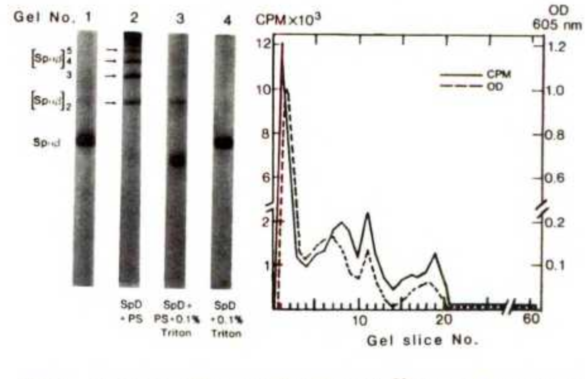

Fig 6. Spectrin dimer association with ^{14}C-phosphatidylserine (PS) liposomes by non-denaturing gel electrophoresis. Spectrin dimers (10 ug) were incubated in 100 uL of 5 mmol/L TES (pH 7.1), 0.15 mol/L NaCl, 0.1 mmol/L EDTA, and 0.1 mmol/L DTT at 0 °C for two hours with 100 ug ^{14}C-phosphatidylserine liposomes, followed by electrophoresis for 22 hours in the absence of SDS, as described in Methods. Control samples with SpD-PS or pure SpD were incubated with 0.1% Triton-X-100 for an additional ten minutes (before electrophoresis). (A) Coomassie stained gels. Gel 1, spectrin dimers: gel 2, spectrin dimers and PS liposomes; gel 3, spectrin dimers and PS vesicles treated with Triton-X-100; gel 4, spectrin dimers treated with Triton-X-100. (B) SpD-PS (gel 2) corresponding to extracted Coomassie blue stain (spectrin) and radioactivity (PS).

DISCUSSION

Previous reports of the interaction of spectrin with the membrane lipids are mainly confined to indirect evidence. These data were obtained through studies on the effect of spectrin on lipid vesicle phase transition,[15] lipid monolayer surface area,[13] lipid bilayer rearrangement,[16,17,20] and identification of hydrophobic domains of this protein.[24] In this report, we provide direct ultrastructural evidence that spectrin interacts with PS vesicles. The ultrastructural results of spectrin-PS interaction suggest that the binding is relatively specific for spectrin, since under the same experimental conditions, actin, ankyrin, and albumin did not associate with PS vesicles. In contrast, protein 4.1 was found to associate with PS vesicles, which is the subject of another communication (Cohen AM, Liu SC, Palek J, manuscript in preparation). We find various regions of the spectrin molecule involved in SpD association with PS vesicles, as documented by the considerable variability of the contact regions (272 ± 144A° from either side of the spectrin molecule). In addition, at higher SpD concentration, SpD can crosslink PS vesicles, suggesting that both ends of the spectrin molecule have binding sites for PS. Spectrin tetramers are shown to bind and crosslink PS vesicles. Thus, the result is consistent with the hypothesis that some of the PS binding sites are located at the distal end region of the spectrin molecule. The exact number of PS binding sites and their location on the spectrin molecule are presently not determined.

In addition to the ultrastructural evidence, we documented the interaction by employing nondenaturing gel electrophoresis. Spectrin dimers incubated with ^{14}C-PS vesicles were separated by nondenaturing gel electrophoresis into multiple high molecular bands. These spectrin high molecular weight bands, as visualized by Coomassie blue absorbance, closely corresponded to the ^{14}C-PS distribution, suggesting that they are complexes of SpD-PS. These bands did not represent oligomeric forms of spectrin, as indicated by their complete solubilization by Triton-X-100.

Spectrin is anchored to the membrane both at its head and tail ends by high affinity interactions with ankyrin and protein 4.1, respectively.[8,11] Based on the direct demonstration of PS-spectrin interaction reported here, as well as previously published, largely indirect data,[14–16,20–22,24] we speculate that additional low affinity interactions of spectrin and possibly other skeletal proteins[35] with lipids of the inner lipid bilayer might exist, thereby further stabilizing the lipid bilayer. Furthermore, the possible biological significance of such low affinity interactions between spectrin and PS is enhanced by the estimated high local concentration of spectrin (230 mg/mL)[3,34,36] close to the inner leaflet of the membrane lipid bilayer. The role of the spectrin-lipid interactions in the maintenance of lipid bilayer stability in certain hemolytic disorders, such as hereditary spherocytosis, which is characterized by membrane instability leading to a loss of membrane lipid,[37,38] remains to be elucidated.

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REFERENCES

1. Shotton DM, Burke BE, Branton D: The molecular structure of human erythrocyte spectrin. Biophysical and electron microscopic studies. J Mol Biol 131 303, 1979

2. Speicher DW, Marchesi VT: Erythrocyte spectrin is comprised of many homologous triple helical segments. Nature 311:177, 1984

3 Morrow JS, Marchesi VT: Self-assembly of spectrin oligomers in vitro: A basis for a dynamic cytoskeleton. J Cell Biol 88:463, 1981

4. Liu SC, Windisch P, Kim S, Palek J: Oligomeric states of spectrin in normal erythrocyte membranes. Biochemical and electron microscopic studies. Cell 37:587, 1984

5. Brenner SL, Korn ED: Spectrin/actin complex isolated from sheep erythrocytes accelerates actin polymerization by simple nucleation. J Biol Chem 255:1670, 1980

6. Cohen CM, Foley SF: Spectrin-dependent and independent association of F-actin with the erythrocyte membrane. J Cell Biol 86:694, 1980

7. Ungewickell E, Bennet PM, Calvert R, Ohanian V, Gratzer WB: In vitro formation of a complex between cytoskeletal proteins of the human erythrocyte. Nature 280:811, 1979

8. Tyler JM, Hargreaves WR, Branton D. Purification of two spectrin-binding proteins Biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1 Proc Natl Acad Sci USA 76:5192, 1979

9. Cohen CM, Korsgren C: Band 4.1 causes spectrin-actin gels to become thixiotropic. Biochem Biophys Res Commun 97:1429, 1980

10. Fowler V, Taylor DL. Spectrin plus band 4.1 cross-link actin, regulation by micromolar calcium. J Cell Biol 85:361, 1980

11. Bennett V, Stenbuck PJ: The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. Nature 280 468, 1979

12. Anderson RA, Lovrien RE: Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. Nature 307:655, 1984

13. Pasternack GR, Anderson RA, Leto TL, Marchesi VT: Interactions between protein 4.1 and band 3, an alternative binding site for an element of the membrane skeleton. J Biol Chem 260:3676, 1985

14. Mombers C, De Gier J, Demel RA, Van Deenen LLM: Spectrin-phospholipid interaction A monolayer study. Biochim Biophys Acta 603:52, 1980

15. Mombers C, Verkleiz AJ, DeGier J, Van Deenen LLM: The interaction of spectrin-actin and synthetic phospholipids. The interaction with phosphatidylserine. Biochim Biophys Acta 551:271, 1979

16 Haest CWM, Plasa G, Kamp D, Deuticke B: Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. Biochim Biophys Acta 509:21, 1978

17 Schwartz RS, Duzgunes N, Chiu D T-Y, Lubin B Interaction of phosphatidylserine-phosphatidylcholine liposomes with sickle erythrocytes. Evidence for altered membrane surface properties. J Clin Invest 71:1570, 1983

18. Bergmann WL, Dressler V, Haest CWM, Deuticke B Crosslinking of SH-groups in the erythrocyte membrane enhances transbilayer reorientation of phospholipids. Biochim Biophys Acta 769:390, 1984

19. Mohandas N, Wyatt J, Mel SF, Rossi ME, Shohet SB Lipid translocation across the human erythrocyte membrane, regulatory factors. J Biol Chem 257:6537, 1982

20. Lubin B, Chiu D. Membrane phospholipid organization in pathologic human erythrocytes, in Sheppard JR, Anderson E, Eaton JW (eds): Membranes and Genetic Disease. New York, Liss, 1982, p 137

21. Schwartz RS, Chiu DT-Y, Lubin B Plasma membrane phospholipid organization in human erythrocytes. Current Topics in Hematol 5.63, 1985

22. Williamson P, Bateman J, Kozarsky K, Mattocks K: Involvement of spectrin in maintenance of phase-state asymmetry in the erythrocyte membrane. Cell 30 725, 1982

23 Isenberg H, Kenna JG, Green NM, Gratzer WB: Binding of hydrophobic ligands to spectrin FEBS Lett 129:109, 1981

24 Bonnet D, Begard E: Interaction of anilinonaphtyl labeled spectrin with fatty acids and phospholipids: A fluorescence study. Biochem Biophys Res Comm 120.344, 1984

25. Cohen AM, Liu SC, Palek J: Biochemical and ultrastructural studies of the interaction of spectrin with phosphatidylserine liposomes. Blood 64:25a, 1984 (abst R-M)

26 Dodge JT, Mitchell C, Hanahan DJ. The preparation and

chemical characteristics of hemoglobin free ghosts of human erythrocytes. Arch Biochem Biophys 100·119, 1963

27. Szoka F, Papahadjopoulos D· Comparative properties and methods of preparation of lipid vesicles (liposomes). Ann Rev Biophys Bioeng 9:467, 1980

28. Ames BN Assay of inorganic phosphate, total phosphate and phosphatases. Methods in Enzymol 8.115, 1962

29. Tyler JM, Branton D. Rotary shadowing of extended molecules dried from glycerol J Ultrastruc Res 71·95, 1980

30 Granger BL, Repasky EA, Lazarides E: Synemin and vimentin are components of intermediate filaments in avian erythrocytes. J Cell Biol 92.299, 1982

31 Liu SC, Palek J, Prchal J, Castleberry RP: Altered spectrin dimer-dimer association and instability of erythrocyte membrane skeletons in hereditary pyropoikilocytosis. J Clin Invest 68:597, 1981

32. Cohen CM, Jackson PL, Branton D: Actin-membrane interactions· Association of G-actin with the red cell membrane. J Supramol Struct 9·113, 1978

33 Morrow JS, Speicher DW, Knowles WJ, Hsu CJ, Marchesi VT Identification of functional domains of human erythrocyte spectrin Proc Natl Acad Sci USA 77:6592, 1980

34. Morrow JS, Haigh WB, Marchesi VT: Spectrin oligomers· A structural feature of the erythrocyte cytoskeleton. J Supramol Struct 17:275, 1981

35. Sato SB, Ohnishi S: Interaction of a peripheral protein of the erythrocyte membrane, band 4.1, with phosphatidylserine-containing liposomes and erythrocyte inside-out vesicles. Eur J Biochem 130:19, 1983

36. Lux SE· Spectrin-actin membrane skeleton of normal and abnormal red blood cells Semin Hematol 16.21, 1979

37. Reed CF, Swisher SN: Erythrocyte lipid loss in hereditary spherocytosis. J Clin Invest 45:777, 1966

38 Johnson R. Red cell membrane proteins and lipids in spherocytosis. Scand J Haematol 20.341, 1978

39. Mombers CAM: Investigations on spectrin-lipid interaction in model systems. Dissertation at the University of Utrecht, 1982

Platelet-Collagen Interaction: Inhibition by Ristocetin and Enhancement by von Willebrand Factor–Platelet Binding

By Frank M. LaDuca, Richard E. Bettigole, William R Bell, and Elean B. Robson

The contribution of von Willebrand factor (vWF)–platelet binding to platelet-collagen interaction was examined in vitro. The binding of vWF to platelets was mediated and regulated by ristocetin. Subthreshold concentrations of ristocetin ($\leq$1 mg/mL), insufficient to cause ristocetin-induced platelet aggregation (RIPA), were added to platelet-rich plasma (PRP) prior to the addition of collagen. The collagen-induced platelet aggregation (CIPA) was modified by ristocetin and the degree of alteration was dependent on the ristocetin concentration. Response as a function of ristocetin concentration was designated the Collagen-Platelet Aggregation Response (Col-PAR). In normal PRP the Col-PAR was a progressive inhibition followed by decreasing inhibition and then an enhanced response. The enhanced response occurred over a narrow range of ristocetin concentrations (0.8 to 1.0 mg/mL). In the absence of vWF (severe von Willebrand's disease, Type I, vWF <1%) the Col-PAR was a progressive, eventually complete inhibition with no enhanced response (with ristocetin concentrations up to 3.0 mg/mL). With addition of vWF to this PRP an enhanced response was observed at a ristocetin concentration inversely proportional to the vWF level. PRP from a patient with severe Hemophilia A showed a response within the normal range. Subthreshold ristocetin did not cause plasma protein precipitation or platelet release of ^{3}H-serotonin, nor induce micro platelet aggregate formation. Digestion of platelet membrane glycoproteins (GP(s)) with chymotrypsin demonstrated that upon removal of GPI, RIPA was absent, CIPA retained and the Col-PAR was progressive inhibition, with no enhancement. With removal of GPs I, II, and III, RIPA, CIPA, and the Col-PAR were absent. A dose-response ^{125}I-vWF-platelet binding occurred with increasing ristocetin concentrations which was unchanged by the addition of collagen. These results demonstrated that ristocetin-platelet association inhibited CIPA, and vWF-platelet binding enhanced platelet-collagen adhesion and platelet aggregation. The in vitro–enhanced CIPA represents a vWF-dependent aggregation of sufficient magnitude to overcome the inhibitory effect of ristocetin. These studies demonstrate an influential interaction of ristocetin, vWF, and collagen with the platelet membrane and imply an important hemostatic contribution of vWF-platelet binding in platelet-collagen interaction.

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At sites of vascular injury platelets adhere to the collagenous vascular subendothelium and subsequently aggregate forming a platelet plug, an integral component of hemostasis.[1] Normal platelet-subendothelial adherence requires von Willebrand factor (vWF), a subunit of the Factor VIII/vWF complex.[2,3] In vitro platelet adherence to deendothelized vessels[4,5] and platelet adhesion to collagen fibrils[6–8] are also vWF-dependent phenomena.

vWF is also required for ristocetin-induced platelet aggregation (RIPA).[9] Ristocetin induces aggregation by binding to platelets[10–12] mediating the binding of plasma vWF[13,14] to the platelet glycoprotein Ib (GPIb) receptor.[15] The extent of vWF-platelet binding is dependent on the ristocetin concentration[13] requiring a threshold level for aggregation.[16,17] Though no in vivo analogue of ristocetin has been demonstrated, evidence suggests a correlation between in vitro RIPA and in vivo platelet-subendothelial adhesion. In von Willebrand disease (vWD) both platelet functions are abnormal[2–5] due to missing or nonfunctional plasma vWF Furthermore, in patients with Bernard Soulier Syndrome (BSS) the presence of normal vWF is insufficient to support either RIPA[18] or platelet-subendothelial adhesion[19] due to a primary platelet membrane defect[18] which may involve an abnormal GPIb platelet receptor.[20]

The previous studies of the role of vWF in platelet-collagen interaction[4–8] have not evaluated the role of vWF-platelet binding in this response. The abnormalities in vWD and BSS suggest that the contribution of this binding to platelet-collagen interaction is likely significant.

This study utilizes ristocetin-mediated platelet-vWF binding to evaluate the effect of subthreshold vWF-platelet binding on in vitro collagen-induced platelet aggregation At these subthreshold ristocetin concentrations the extent of vWF-platelet binding is proportional to the ristocetin concentration yet insufficient to induce aggregation. In these experiments the aggregation response to collagen was enhanced in the presence of increasing vWF-platelet binding and inhibited in the presence of ristocetin-platelet association. These results provide information on the functional in vitro interaction of ristocetin, vWF, and collagen at the platelet membrane surface.

MATERIALS AND METHODS

Reagents

Ristocetin was purchased as a lyophilized powder containing greater than 90% Ristocetin A, remainder Ristocetin B (Bio Data Corp, Horsham, Pa). Reagent lots used in a given study are identified by manufacturer lot numbers. Stock solutions of 30 mg/mL in 0.85% NaCl were stored at −30 °C, from which working solutions were prepared and kept on ice during the study period Soluble calf skin collagen (Type III; 7 8 mg/mL) (Worthington Division, Millipore Corp, Freehold, NJ) was prepared by sodium acetate extraction of fresh calf skin.[21] The resulting collagen preparation, which is soluble in salt solutions, was diluted in 0 9% NaCl

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(concentration = 1,950 µg/mL) and stored at 4 °C for use within 1 week Fibrillar collagen was kindly provided by Carolyn Floyd (George Washington University, Washington, DC). It was prepared from acid-soluble calf skin collagen (Type III, Sigma Chemical Co, St. Louis, Mo) by extraction with 0.5 mol/L acetic acid at 4 °C, precipitation with 4% NaCl and centrifugation, by the method described [22] The final salt-free monomeric collagen, containing no noncollagenous protein,[22] was lyophilized and stored in liquid nitrogen

Alpha-chymotrypsin (59 U/mg, 3 times recrystallized), soybean trypsin inhibitor (SBTI), phenylmethylsulfonyl flouride (PMSF), adenosine-5′-diphosphate, sodium salt (ADP), bovine serum albumin (BSA), periodic acid, Schiff's reagent, and TRIS (hydroxy methyl-aminomethane) were from Sigma Chemical Co. Chymotrypsin (25 to 1000 µg/mL), SBTI (10 mg/mL), and ADP (200 µmol/L) were dissolved in 0.9% NaCl and stored at −30 °C PMSF (100 mmol/L) was dissolved in 95% ethanol (stored at −30 °C). Acrylamide, bisacrylamide, TEMED, sodium dodecyl sulfate (SDS), and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were from Bio-Rad Labs (Rockville Center, NY). Serotonin (^{3}H-5-Hydroxytryptamine, creatine sulfate, 30 Ci/mmol) was obtained from New England Nuclear (E I duPont de Nemours and Co, Boston, Mass) Carrier-Free Na ^{125}I (3.7 GBq ^{125}I/mL) was from Amersham Corp (Arlington Heights, Ill). All other chemicals were of reagent grade and obtained from Fisher Scientific Co (Fairlawn, NJ). All containers, tubes, and pipettes used in preparation and utility of plasma samples were plastic.

Blood Collection, Preparation of Platelet-Rich and Platelet-Poor Plasma

Blood was obtained after informed consent according to the guidelines of the Human Investigational Committee from healthy, normal donors and study subjects from an antecubital vein using a 19-gauge or 21-gauge butterfly needle. A two-syringe technique was employed As citrate concentration affects platelet function,[23] the blood was anticoagulated with 1/12 volume 3.8% sodium citrate and corrected to a standard final citrate concentration of 20 0 ± 0.1 mmol/L in the platelet-rich plasma (PRP) and platelet-poor plasma (PPP). Citrate correction was done by determination of the [citrate/plasma] volume ratio based on the donor hematocrit (corrected for citrate addition) as determined on a Coulter S Plus Two Counter (Coulter Electronics, Inc, Hialeah, Fla). PRP was prepared by centrifugation of whole blood at 180 × g for 15 minutes at 22 °C. PPP was prepared by centrifugation at 1800 × g for 20 minutes at 22 °C. The platelet count of the PRP was determined on a Coulter S Plus Two Counter and adjusted to 300,000/µL by addition of an appropriate volume of PPP. Random platelet counts showed good correlation with those obtained on a Thrombocounter (Coulter Model ZBI) and with a manual technique (Neubauer improved hemocytometer).

Heparinized PRP was prepared from whole blood that was anticoagulated with 1 unit of heparin/mL (Heparin sodium, from porcine intestinal mucosa, A.H. Robbins Corp, Richmond, Va).

Normal Pooled Plasma (NPP), used for assay of plasma factors, was obtained from 20 healthy individual donors aged 20 to 40 years who were taking no medications Blood was collected in 1/10 volume 3.8% sodium citrate using a two-syringe technique, centrifuged at 2000 × g for 15 minutes at 4 °C, and equal volumes of PPP from each sample were pooled and the pooled plasma frozen at −70 °C. No citrate correction was necessary as this PPP was not used in platelet aggregation tests.

Platelet Aggregation

Platelet aggregation was performed at 37 °C on platelet count-adjusted, citrate-adjusted PRP in one of two dual-channel aggregometers (Chronolog Corp, Havertown, Pa) using the turbidimetric method.[24] Light transmission was recorded on a dual-channel chart recorder (Riken Denshi Recorder, Payton Associates, Buffalo, NY) with a chart rate of 20 mm/min This allowed for calculation of aggregation response versus time of aggregation reaction. To minimize pH changes in plasma due to CO_2 diffusion with time, the pooled PRP was aliquoted into smaller (5 mL and 10 mL) capped plastic tubes [25] PRP samples were withdrawn quickly and the tubes recapped. In our test system, four PRP samples were evaluated simultaneously, enabling a completion of aggregation tests within 75 to 90 minutes of PRP preparation. With these procedures the pH of representative PRP samples ranged from 7 52 to 7 65 (Radiometer Copenhagen, PHM62 standard pH meter with combination glass electrode) during the study period, values within the recommended range [25]

Effect of Ristocetin on Collagen-Induced Platelet Aggregation

In normals, 25 µL of ristocetin, at final concentrations of 0 0 to 1.0 mg/mL, were added to 225 µL of stirred PRP in the aggregometer. In experiments in which PRP contained VWF in amounts less than 50% of normal, ristocetin was also used in final concentrations up to 3.0 mg/mL in addition to these concentrations. After two minutes, ample time for maximal ristocetin-mediated vWF-platelet binding,[13] 25 µL of soluble calf skin collagen was added (final concentration 175 µg/mL) and the platelet aggregation response recorded (Fig 1).

Identical tests were performed in heparinized PRP to evaluate the response in a system unresponsive to ristocetin [26,27] Tests were also performed substituting ADP (final concentrations of 0.5 to 2 5 µmol/L) for collagen.

Analysis of Platelet Aggregation Response

The collagen-induced aggregation curve was assessed by the lag phase (L), reaction rate (Vmax), and the maximum extent of aggregation during the first 7½ minutes of response (Hm) [28] The contribution of these variables to the aggregation response was quantitated by the area under the curve (Fig 1), a measurement we have determined to be a sensitive and reproducible measurement of aggregation.[29,30] Each curve is asymmetric, thus aggregation area was computed using a manual technique in which the aggregation curve is traced on calibrated tracing paper, cut out, and weighed on a Mettler Analytical Balance (Will Scientific, Inc, Rochester, NY) Weight is directly proportional to area. All units of area are reported as weight in milligrams. Two aggregation areas based upon different length of time of aggregation were used (Fig 1): (a) four minutes (Area 4) from the addition of collagen, and (b) seven and one-half minutes (Area 7½) from the addition of collagen. Area 4 is a measure of the extent of the lag phase and the Vmax. A prolongation of the lag phase and a decreased Vmax results in a decreased Area 4. A shortened lag phase and increased Vmax results in an increased Area 4. If the lag phase extends beyond four minutes, Area 4 is zero. Area 7½ measures the maximum extent of aggregation.

The collagen-induced aggregation response, assessed by these measurements, plotted as a function of increasing ristocetin concentration added, was defined as the Collagen-Platelet Aggregation Response (Col-PAR) Response in the absence of ristocetin is the baseline collagen aggregation. In control samples, ristocetin at subthreshold concentrations was added to the PRP without subsequent collagen addition. Analysis of the Col-PAR was done by standard analysis of variance (ANOVA) and linear regression [31]

Von Willebrand Factor Requirement

Patients with severe von Willebrand's Disease (vWD), Type I (patient SS), severe Hemophilia A (patient TP), and mild vWD,

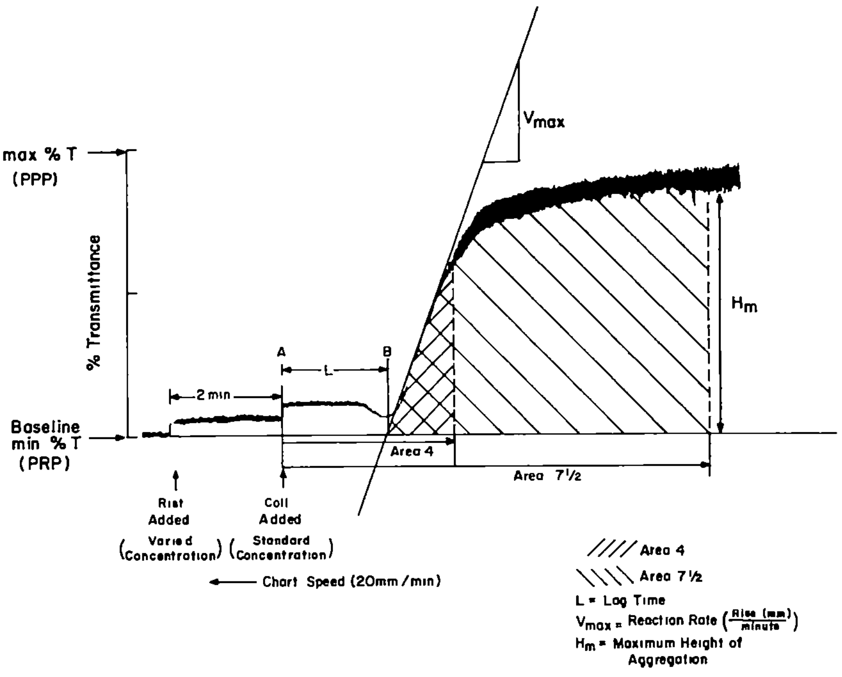

Fig 1. Experimental design and analysis of platelet response. Ristocetin at subthreshold concentrations was added to stirred citrated PRP in the aggregometer two minutes prior to the addition of collagen (final concentration, 175 μg/mL). At these ristocetin concentrations, no aggregation occurs if collagen is not added. The area under the aggregation curve was used to evaluate the lag phase, reaction rate, and maximum extent of aggregation for each aggregation response. The limits of the aggregation area (hatched area) are the aggregation curve, the vertical line from the curve to the baseline at the indicated time, the baseline, and the intercept of the Vmax with the baseline.

Type I (patient DR), were examined to determine the requirement for vWF in the Col-PAR. The hemostatic profiles of these patients are shown in Table 1 At the time of evaluation no patient had received recent transfusions or medications. No inhibitor activity against any Factor VIII subunit was detectable. The Col-PAR was also determined in PRP samples in which the percent vWF present was controlled by addition of normal control PPP (source of vWF) to the PRP of patient SS in varying proportions to produce the desired vWF levels. PRP and PPP were prepared concomitantly from patient SS and the normal control. Appropriate mixtures yielded PRP samples with platelet count of 300,000/μL and vWF measured as VIIIR:Ag[32] of 32%, 16%, 8%, and 4%.

Effect of Ristocetin on Collagen Fibril Formation

Lyophilized collagen was dissolved in 5 mmol/L acetic acid by stirring overnight at 4 °C. The collagen concentration was determined by optical density at 230 nm using a standard curve determined by polarimetry.[22] Collagen fibrils were formed at 35 °C for 17 hours by the method described[22] using 300 μg/mL collagen in 60 mmol/L N-TRIS-2 aminoethanesulfonic acid (TES) buffer, pH 7.5. Fibril formation was studied in the presence of increasing final concentration of ristocetin (0.3 to 1.5 mg/mL). Following incubation the fibrils were centrifuged and the supernatant assayed for residual (nonfibrillar) collagen by optical density at 230 nm. In control samples 5 mmol/L acetic acid was substituted for TES buffer to determine the background A_{230} in the absence of fibril formation.

Evaluation of Micro Platelet Aggregates With Ristocetin

Ristocetin (50 μL) in final concentrations of 0.0 to 1.4 mg/mL was added to duplicate samples of PRP (450 μL). The capped samples were incubated with mixing at 37 °C for seven minutes After incubation micro platelet aggregate formation was evaluated by (1) fixation of an aliquot of the PRP sample (0.2 mL) with 1.5% formaldehyde (0.2 mL) and estimation of the number of nonaggregated platelets by phase contrast microscopy using a Neubauer improved hemocytometer[33]; (2) preparation of a Wrights stained smear and examination by direct microscopy; and (3) centrifugation of the PRP sample at 80 x g for two minutes and determination of the residual platelet count using a Coulter Counter Model B.[34]

Protein Precipitation With Ristocetin

Ristocetin was added to duplicate samples of PRP and PPP and incubated as described above for microaggregate determination. After incubation the samples were immediately transferred to

Table 1. Hemostatic Profile of Patients Used to Evaluate the vWF Requirement in the Col-PAR

									Platelet Profile		
Patient	Diagnosis	BT	PLT CT	PT	APTT	VIII:C	VIII Ag	VIIIR.WF	RIPA	CIPA	AIPA
SS	vWD (Type 1) severe	>20	310,000	WNL	78.0	3	<1	<1	ABNL	WNL	WNL
DR	vWD (Type 1) mild	WNL	265,000	WNL	WNL	28	42	38	ABNL	WNL	WNL
TP	classical Hemophilia A	WNL	255,000	WNL	90 0	<1	168	185	WNL	WNL	WNL
Normal range		<9	150-350,000	11-13	<40	50-200	50-200	50-200	WNL	WNL	WNL

Abbrev BT, bleeding time (min); PLT CT, platelet count (per mm^3), PT, prothrombin time (sec); APTT, activated partial thromboplastin time (sec); RIPA, ristocetin-induced platelet aggregation, CIPA, collagen-induced platelet aggregation, AIPA, ADP-induced platelet aggregation; WNL, within normal limits, and ABNL, abnormal.

The Factor VIII/vWF complex consists of two moieties with three identifiable properties.[9] In the text VIII:C is the coagulant activity deficient in hemophilia A and vWF is the high molecular weight moiety of the Factor VIII/vWF complex necessary for platelet-related functions and abnormal to various degrees in vWD.[3] It is determined antigenically as VIIIR·Ag, and functionally by ristocetin cofactor activity referred to as VIIIR:WF (VIII C, VIIIR:Ag, and VIIIR:WF expressed as percent activity 100% = 1 U/mL.)

eppendorf micro test tubes, capped, and centrifuged at 8,800 × g for two minutes (Eppendorf Micro Centrifuge). The supernatant was withdrawn and diluted 1 20 in 0.9% NaCl and protein determined by the method of Lowry.[35]

Platelet Release Studies Using ^{3}H-Serotonin (^{3}H-5HT)

Platelets in citrated PRP (platelet count of 450,000/μL) were labeled with ^{3}H-5HT (0.6 μCi/mL) as previously described.[36] The labeled platelets were sedimented on a BSA density gradient and resuspended in calcium-free Tyrodes buffer.[37] Platelet release of ^{3}H-5HT (measured on a Beckman LS-133 Liquid Scintillation Counter) was determined following addition of ristocetin, collagen, or ADP to a mixture of the BSA-washed platelets and normal PPP by the method described.[36] All tests were conducted in triplicate. The percent ^{3}H-5HT release was calculated from the maximal obtainable release of solubilized labeled platelets minus the background supernatant count.[36]

Treatment of Platelets with Chymotrypsin

Platelets in citrated PRP (platelet count of 450,000/μL) were incubated with chymotrypsin (25 to 1000 μg/mL) for 10 to 40 minutes by the method described [38] Following incubation the reaction was stopped by addition of excess SBTI[38] and PMSF [39] The chymotrypsin-treated platelets were sedimented, washed,[37] and resuspended in normal PPP. Aggregation studies were performed as described earlier in the text Additional samples of chymotrypsin-treated platelets were centrifuged (8,800 × g Eppendorf Micro Centrifuge), solubilized in a nonreducing sample buffer (containing 3% SDS), and analyzed by SDS-PAGE by the method of Fairbanks.[40] Gels were stained with Coomassie blue and by the periodic acid-Schiff procedure (PAS).[40]

Von Willebrand Factor–Platelet Binding Studies

vWF was kindly provided by Carolyn Floyd (George Washington University, Washington, DC). It was prepared from cryoprecipitate of fresh frozen plasma by the previously described method [22] Purified vWF (80 units VIIIR WF activity/mg) was radioiodinated in the presence of lactoperoxidase and glucose oxidase using Enzymobeads (BioRad), 70 μg vWF and 1 mCi Na ^{125}I [14] Free ^{125}I was separated from protein-bound iodine on a G-25 (Pharmacia Chemicals, Piscataway, NJ) column using a 20 mmol/L TRIS, pH 7 6, 1 mmol/L EDTA, 100 mmol/L NaCl buffer. Specific activity of the ^{125}I-VWF was 0.2 to 0.3 μCi/μg with no loss of VIIIR:WF activity Washed platelets (WP) obtained by centrifugation of fresh citrated PRP or recently expired platelet concentrates were resuspended in calcium-free Tyrodes buffer containing 5 mmol/L EDTA [37] Binding assays were performed by modification of the described technique[14] with a mixture of 0.3 μg ^{125}I-vWF (024 units VIIIR:WF), 1.6 × 10^{8} WP, and ristocetin in final concentrations up to 1.5 mg/mL. After five minutes (37 °C), 0.9% NaCl was added to one set of assay tubes and collagen (final concentration of 175 μg/mL) to a second set. Following incubation (30 minutes, 37 °C, with stirring) the platelets were sedimented through an oil mixture (N-butyl ester, Bis(−)pthalate ester, 1 1 [vol/vol])[41] using an Eppendorf Micro Centrifuge and the radioactivity of the platelet pellet counted (Beckman Gamma 8000 Counter). All tests were conducted in triplicate.

Assay of Factor VIII–Related Properties

Factor VIII coagulant (VIII:C) was assayed based on a one-stage assay[42] using known substrate deficient plasma from a severe Hemophilia A patient Ristocetin Cofactor Activity (VIIIR:WF) was measured using formalin fixed washed normal platelets and patient plasma Quantitation was based on agglutination rate upon addition of ristocetin [43] vWF was measured immunologically as VIIIR:Ag by quantitative immunoelectrophoresis[32] using rabbit antisera to human Factor VIII–associated protein (Calbiochem-Behring Corp, La Jolla, Calif) and a 5.6 mmol/L barbital, 3 15 mmol/L barbital sodium, 375 mmol/L glycine, 186.6 mmol/L Tris, pH 8.9, buffer Ten μL samples of plasma, undiluted and serially diluted 1.2 in 0.9% NaCl, were applied to a 1% agarose (Marine Colloids Division, FMC Corp, Rockland, Me) gel. Quantitation was based on rocket height In all assays serial twofold dilutions of NPP were used to generate the standard curve. Screening for inhibitors of Factor VIII–related properties was done by assay of residual factor activity in mixtures of test plasma and NPP.[44]

RESULTS

Effect of Ristocetin-Mediated Platelet-vWF Binding on Collagen-Induced Platelet Aggregation

Collagen-Platelet Aggregation Response (Col-PAR). In normal individuals the Col-PAR was a progressive inhibition of aggregation followed by decreasing inhibition and then enhanced responses (Fig 2). In the absence of ristocetin the platelet response was a typical collagen-induced platelet aggregation (Fig 2A) demonstrating the characteristic lag phase, shape change, and aggregation wave. Low ristocetin concentrations (0.2 to 0.6 mg/mL) resulted in an inhibited response (Fig 2B) in which the lag phase was prolonged, the shape change was delayed, Vmax was diminished, and the maximum extent of aggregation was decreased. As ristocetin concentration was increased (0.7 to 1.0 mg/mL), inhibition

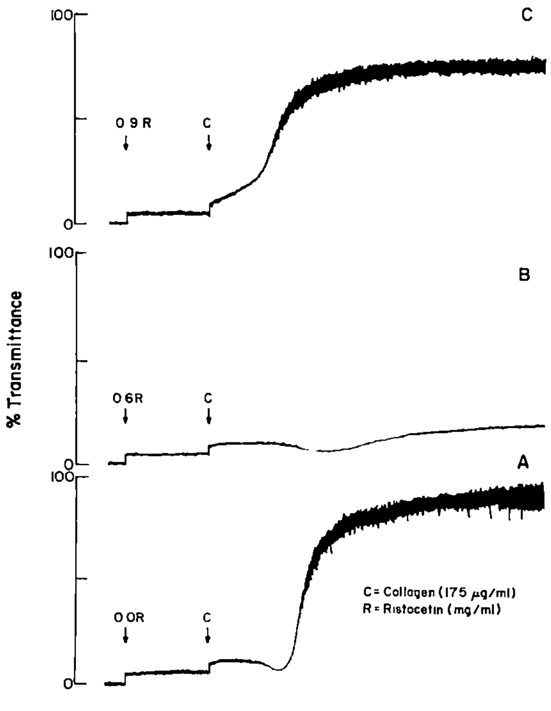

Fig 2. Effect of increasing concentrations of ristocetin on collagen-induced platelet aggregation in PRP from a normal individual. Drawings are actual aggregometer tracings. Experimental design as shown in Fig 1. (A) Baseline response (diluent added); normal collagen-induced aggregation. (B) Inhibited response (ristocetin concentration of 0.6 mg/mL); prolonged lag phase, diminished aggregability (C) Enhanced response (ristocetin concentration of 0.9 mg/mL); shortened lag phase, increased platelet aggregability compared to inhibited response.

was overcome resulting in an enhanced response (Fig 2C). The enhanced response represents that collagen-induced aggregation in which the lag phase is considerably shortened compared to the control collagen response (ie, response in the absence of ristocetin) and the Vmax and Hm are increased from the inhibited response.

The aggregation area quantitated the degree of inhibition and enhancement (Fig 3). Area 4, most sensitive to the lag phase, demonstrated the significant prolongation and shortening of the lag phase at inhibited and enhanced responses, respectively. Area 7½ reflected the decreased maximum extent of aggregation with inhibition and the increased response as ristocetin concentration was increased. In these 25 normal subjects studied with Ristocetin lot 036101A maximal inhibition occurred at a ristocetin concentration of 0.6 mg/mL and increasing responses at concentrations of 0.7 mg/mL and greater. Twenty-two subjects demonstrated an enhanced response at ristocetin concentrations of 0.7 to 1.0 mg/mL. At these ristocetin concentrations no measurable aggregation was seen if collagen was not added. At ristocetin concentrations greater than those resulting in an enhanced response, the addition of ristocetin resulted in RIPA. This range of RIPA was compatible with previous studies in our lab of normals and abnormals using this ristocetin lot. In individual subjects, when the enhanced response did not occur until a concentration of 1.0 mg/mL, the inhibition of the aggregation response increased progressively with increasing ristocetin concentrations of 0.7 to 0.9 mg/mL.

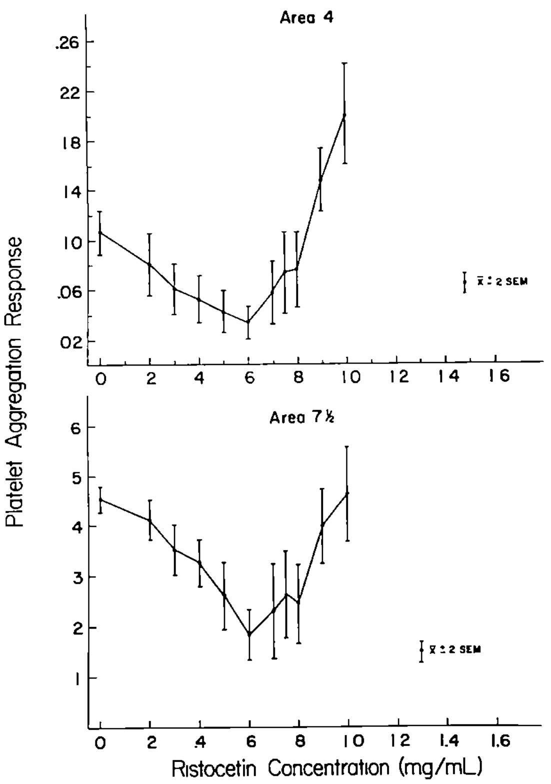

Fig 3. The Collagen-Platelet Aggregation Response (Col-PAR) is the collagen-induced platelet aggregation as a function of ristocetin concentration added. Values shown are for 25 normal individuals using Ristocetin lot 036101A. Increasing ristocetin concentration from 0.0 to 1.0 mg/mL results in a progressive inhibition of response followed by decreasing inhibition and then enhanced responses. The four-minute area (Area 4) measures the change in lag phase and Vmax; the seven and one-half-minute area (Area 7½) measures the change in the maximum extent of platelet aggregability. Aggregation area expressed as weight, in milligrams, of aggregation tracings ±2 standard error of the mean (SEM). The aggregation area at varying ristocetin concentrations is statistically different ($P < .01$, ANOVA analysis). The same response was obtained with four additional ristocetin lots.

Col-PAR in heparinized PRP. Platelets in heparinized PRP aggregated in response to ADP and collagen but not to ristocetin. In heparinized samples the Col-PAR demonstrated no inhibition of collagen response at a ristocetin concentration of 0.6 mg/mL nor enhanced responses at a ristocetin concentration of 1.0 mg/mL, concentrations that produced inhibited and enhanced responses in citrated PRP.

Effect of ristocetin on ADP-induced platelet aggregation. In the absence of ristocetin, a biphasic aggregation response was obtainable with an ADP concentration of 1.25 μmol/L to 2.5 μmol/L. The addition of ristocetin to PRP (0.0 to 1.0 mg/mL) prior to ADP addition (1.25 to 2.5 μmol/L) resulted in (1) no inhibition of ADP-induced aggregation (ristocetin concentration ≤0.7 mg/mL) and (2) an enhanced secondary aggregation wave at ristocetin concentrations (0.8 to 1.0 mg/mL) corresponding to an enhanced collagen response in the Col-PAR. At low ADP concentrations (≤1.0 μmol/L) not producing a secondary aggregation wave in the absence of ristocetin, no enhanced response upon addition of ristocetin was observed.

Effect of Ristocetin on Collagen Fibril Formation

Following the addition of TES buffer to soluble collagen, ristocetin diminished the extent of collagen fibrillar formation as measured by an increased residual soluble collagen in the supernatant. Inhibition in the extent of fibril formation was identical at ristocetin concentrations of 0.3 to 1.5 mg/mL.

Protein precipitation with ristocetin. In a normal donor plasma sample, total protein was assayed at 7.4 g/dL Upon addition of ristocetin (concentrations to 1.4 mg/mL), no appreciable loss of protein, due to precipitation, was observable in the supernatant (<5% of the starting protein value). No difference of protein recovery was observed between PRP and PPP.

Evaluation of Micro Platelet Aggregation With Ristocetin

No micro platelet aggregate formation was demonstrable upon addition of increasing subthreshold concentrations of ristocetin to a normal donor PRP. With threshold ristocetin concentrations (1.0 to 1.4 mg/mL), there was a progressive increase in the formation of platelet aggregates. The Col-PAR of this donor PRP demonstrated an inhibited response at 0.8 mg/L, an enhanced response at 0.9 mg/mL, and minor RIPA at 1.0 mg/mL. The first signs of platelet aggregate formation corresponded to the ristocetin concentration producing the first (minor) RIPA in the aggregometer.

Phase contrast evaluation of fixed PRP samples. With ristocetin concentrations <1.0 mg/mL all platelets were single (>95% nonaggregated). At 1.0 mg/mL the PRP is a heterogenous mixture of single platelets, platelet doublets, and small aggregates. At concentrations >1.0 mg/mL, there are large platelet aggregates (95% aggregated at 1.4 mg/mL).

Residual platelet count determinations in PRP. Platelet counts following ristocetin addition and subsequent centrifugation to remove small aggregates showed no change of platelet count at concentrations <1.0 mg/mL. At 1.0 mg/mL a 40% decrease in platelet count occurred that increased proportionately with the increase of ristocetin concentration to 1.4 mg/mL.

Direct microscopy of Wright's stained smears. Single platelets were seen at ristocetin concentrations <1.0 mg/mL, a heterogenous mixture of single platelets, doublets, and small aggregates at 1 0 mg/mL, and large clumps at 1.4 mg/mL

Platelet Release of ^{3}H-5HT

Subthreshold ristocetin not causing measurable RIPA did not induce a release of ^{3}H-5HT from labeled platelets (<5% release measurable in the supernatant). Furthermore, at threshold ristocetin concentrations measurable release of ^{3}H-5HT did not occur with minor monophasic RIPA (<5% at ristocetin concentrations of 0.9 to 1.1 mg/mL), and was measurable only when RIPA resulted in the secondary aggregation wave (57% release at 1.5 mg/mL). The secondary aggregation wave induced by ADP, and aggregation induced by collagen, caused measurable ^{3}H-5HT release.

Treatment of Platelets With Chymotrypsin: Effect on the Col-PAR

Platelets treated with chymotrypsin and analyzed by SDS-PAGE demonstrated loss of PAS staining material, identifiable by molecular weight as platelet membrane glycoproteins (GPs) (according to the nomenclature of Phillips and Agin[45]). The extent of GP digestion was dependent on the chymotrypsin concentration. At 500 μg/mL the removal of GPI resulted in the loss of RIPA and retention of collagen-induced aggregation; the Col-PAR was a progressive inhibition without enhancement. At higher chymotrypsin concentration (1000 μg/mL), platelet GPs I, II, and III were not identifiable by PAS staining, resulting in the loss of RIPA, collagen-induced aggregation, and the Col-PAR (Table 2).

von Willebrand Factor Requirement

Severe von Willebrand's disease In the absence of vWF (PRP from patient SS), the Col-PAR was a progressive inhibition with no subsequent enhanced response with ristocetin concentrations up to 3.0 mg/mL (Fig 4). With increasing ristocetin concentration the lag phase was progressively prolonged (Area 4) and the maximum extent of aggregation totally inhibited (Area 7½). No protein precipitation was observed at these ristocetin concentrations.

Addition of von Willebrand factor. As vWF was added to the PRP lacking it an enhanced response was observed (Fig 5). The ristocetin concentrations at which an enhanced response was seen was inversely proportional to the amount of vWF present. With increasing vWF level, the Col-PAR approached the normal range and the control response. The normal range consisted of individuals with VIIIR:Ag levels of 49% to 202% and all demonstrated enhanced responses with ristocetin concentrations of 1.0 mg/mL or less (shaded area, Fig 5) All vWF mixtures studied had abnormal VIIIR:Ag levels (32%, 16%, 8%, 4%) and the Col-PAR fell outside of the normal range, none producing an enhanced response at ristocetin concentration of 1.0 mg/mL. The progressive inhibition of Area 7½ reflects the increasing inhibition of the maximum extent of aggregation with increasing ristocetin concentration. In all samples with vWF levels less than 50%, the collagen response in the absence of ristocetin was similar to that of normal individuals (Ristocetin at 0.0 mg/mL: Figs 4 and 5).

In the absence of Factor VIII coagulant but normal vWF (patient TP; classical hemophilia), the Col-PAR was within the normal range with an enhanced response at a ristocetin concentration of 0.9 mg/mL. With diminished vWF (patient DR; mild vWD) tests, on two separate occasions, demonstrated a response outside the normal range with an inhibited response at 1.0 mg/mL and an enhanced response at 1.1 mg/mL.

von Willebrand Factor Platelet Binding

Increasing amounts of ^{125}I-vWF bound to platelets as the ristocetin concentration was increased to a final concentration of 1.5 mg/mL (Fig 6). In the absence of ristocetin a small amount of vWF-platelet binding occurred (<6% maximal). The dose-response increase of vWF-platelet binding with increased ristocetin concentration was unchanged using

Table 2. Effect of Chymotrypsin Removal of Platelet Glycoproteins (GPs) on Platelet Aggregation Response

Treatment (chymotrypsin, μg/mL)	GP(s) Present (SDS-PAGE and PAS Stain)	Aggregation Response		
		RIPA	CIPA	Col-PAR
0 (control)	I, II, III, IV	NL	NL	inhibition-enhancement
500	II, III, IV	absent	NL	inhibition
1000	IV	absent	absent	absent

Abbrev: RIPA, ristocetin-induced platelet aggregation, CIPA, collagen-induced platelet aggregation, Col-PAR, Collagen-Platelet Aggregation Response, and NL, normal

Platelets were incubated with increasing concentrations of chymotrypsin to limit digest the platelet GPs.[38] Following chymotrypsin treatment one sample of platelets was tested for aggregability and a second sample was analyzed by SDS-PAGE and stained by PAS [40] Platelet GPs were identified by relative migration in the gel based on the known molecular weights.[45] In control samples (no chymotrypsin) the platelets aggregate with ristocetin and collagen and the Col-PAR is inhibition-enhancement. With chymotrypsin at 500 μg/mL, GPI is removed from the platelet, RIPA is lost, CIPA is normal, and the Col-PAR is progressive inhibition (no enhancement) At higher chymotrypsin concentrations (1000 μg/mL), GPI, II, and III are removed and all aggregation response is lost

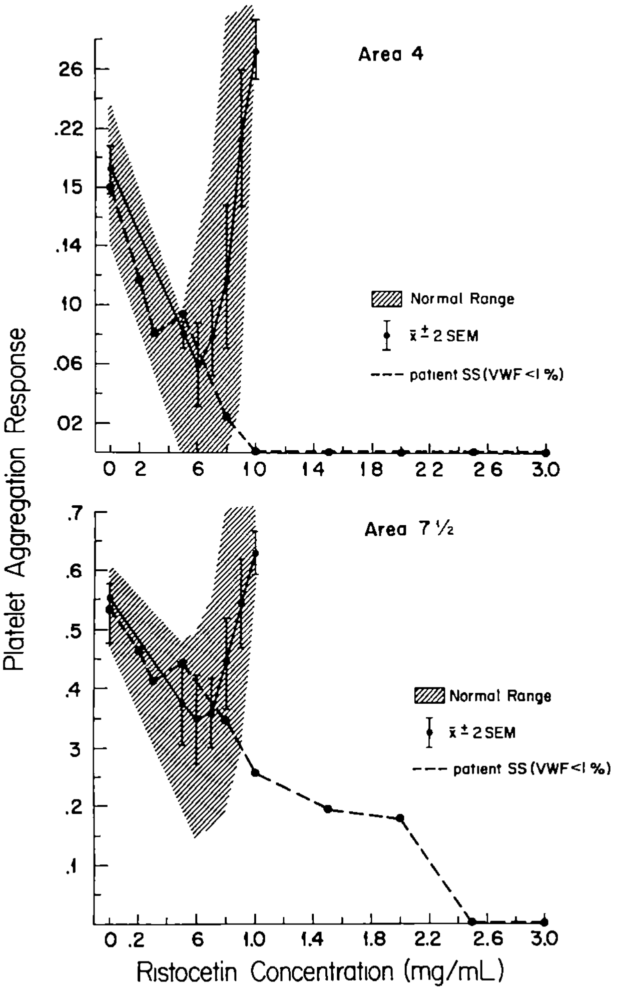

Fig 4. Col-PAR in severe von Willebrand's disease. Type I, (vWF <1%). Shaded area is range of responses from 13 normal individuals (VIIIR:Ag levels of 49% to 202%) evaluated with Ristocetin lot 064014C. Solid line is the mean value of normals ±2 SEM. In the absence of vWF the Col-PAR is a progressive, eventually complete, inhibition. The limit of Area 4 is reached when the lag phase extends beyond four minutes. A similar response was observed on four separate test occasions and with different ristocetin lots.

different vWF concentrations (0.15 to 0.45 μg). No increased binding occurred in the presence of collagen using WP from either fresh or expired platelet concentrates.

Statistical model. The Col-PAR was statistically analyzed by linear regression (ANOVA). In this model the aggregation responses obtained for a given individual at given ristocetin concentrations were assigned numerical codes based on those responses being: (1) progressively inhibited, (2) maximally inhibited, (3) progressively increasing, and (4) maximally enhanced. The aggregation responses are expressed as a function of the summation of the percent vWF and the ristocetin concentrations resulting in inhibition and enhancement, respectively. Statistical analysis of the linear regression of response (ie, area) versus ristocetin concentration determined the applicability of defining the Col-PAR by an inhibition-enhancement model. The calculated F ratios verified the statistical significance of defining the Col-PAR by inhibition-enhancement as a function of ristocetin concentration ($P < .00001$). The difference between groups of normals and abnormals (ie, vWF <49%) was statistically significant ($P < .00001$).

DISCUSSION

In vitro platelet-collagen interaction has been shown to be a vWF-dependent response,[6-8] yet the nature of the vWF requirement is poorly defined. To characterize the vWF role we have determined the effect of ristocetin-mediated platelet-vWF binding on collagen-induced platelet aggregation. Our results demonstrate that ristocetin-platelet association inhibits the platelet aggregation response to collagen and vWF-platelet binding enhances collagen-induced platelet aggregation.

Ristocetin binds to platelets[10-12] inducing a concentration-dependent vWF-platelet binding at GPIb.[13-15] In our studies, as confirmed by radiolabeled vWF, the extent of vWF-platelet binding was regulated by control of the ristocetin concentration. Collagen-induced aggregation was chosen to evaluate the platelet-collagen interaction as it measures both platelet-collagen adhesion and platelet aggregability, and is a useful in vitro test of platelet function.[18,28] Adhesion is characteristic of the lag phase of aggregation during which the platelet membrane is altered, exposing "reactive sites"[1] and promoting platelet aggregation via a release reaction.[28]

With normal vWF level (49% to 202%) the platelet aggregation response to collagen as a function of increasing ristocetin concentration demonstrated two phenomena: inhibition and enhancement. Inhibition, at low ristocetin concentrations, was characterized by decreased adhesion and aggregation (ie, prolonged lag phase and diminished maximum extent of aggregation). At higher ristocetin concentrations, enhancement represented an aggregation response requiring vWF in which adhesion was increased (ie, shortened lag phase) from the control collagen response (ie, no ristocetin) and platelet aggregability was of sufficient magnitude to overcome the inhibitory effect of ristocetin. With abnormal vWF level (≤42%), the inhibition phase was similar yet the enhanced response was a function of the vWF level (Figs 4 and 5). However, regardless of the vWF level, the collagen-induced aggregation in the absence of ristocetin was similar; thus the enhanced response was actually a function of the ability of vWF to bind to platelets in the presence of ristocetin. The correlation between an abnormal vWF level (≤42%) and the ristocetin concentration producing an enhanced response was statistically significant. With normal vWF level (≥49%) the same correlation was evident (ie, normal individual with 49% showed an enhanced response at 1.0 mg/mL, an individual with 202% at 0.8 mg/mL) but the limited data did not lend to the same statistical correlation between high and low normal vWF values.

The mechanism by which ristocetin induces vWF-platelet binding is poorly defined. Ristocetin-platelet association, which is a low affinity, concentration-dependent response, reduces the negative surface charge of the platelet[12,13] exposing the GPIb receptor domain that binds vWF. In our studies the lack of micro platelet aggregate formation and of platelet release of ^{3}H-serotonin in the presence of subthreshold

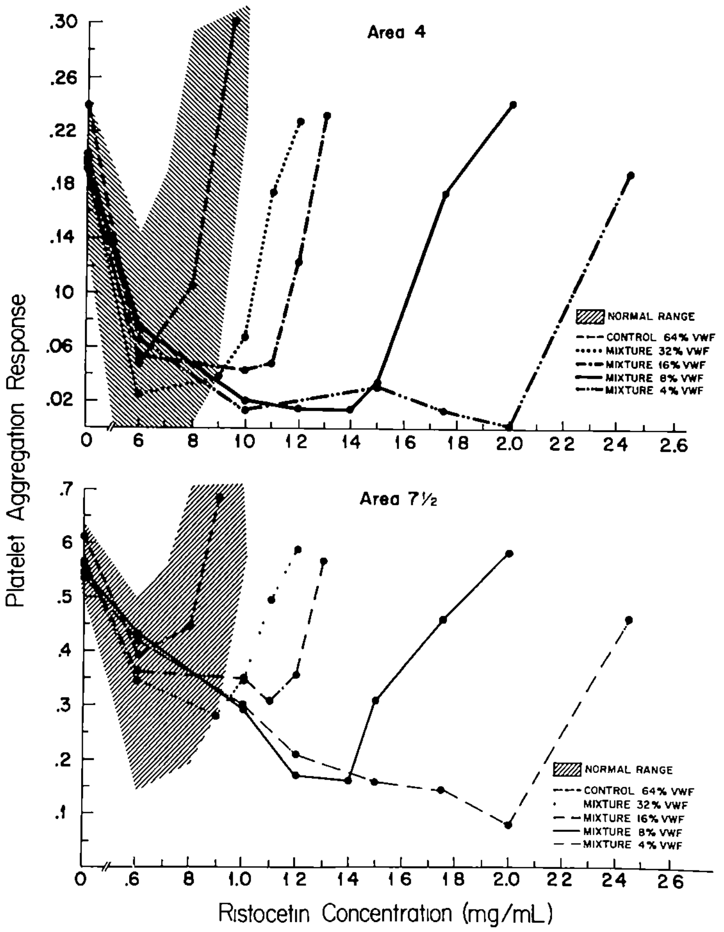

Fig 5. Relationship of vWF level to the Col-PAR. PPP from the normal control (source of vWF) was added to the PRP of a severe vWD patient (patient SS) and the Col-PAR evaluated. Appropriate mixtures yielded samples with platelet count of 300,000/μL and the indicated vWF levels (expressed as VIIIR:Ag). The ristocetin concentration producing an enhanced response was inversely related to the vWF present. Normal range as in Fig 4.

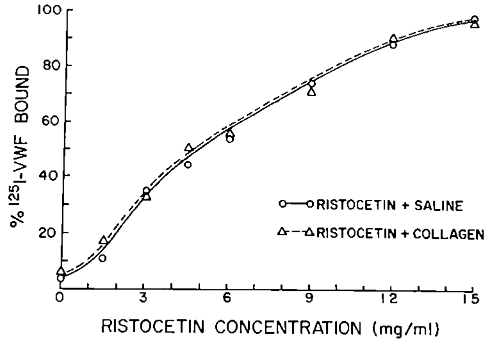

Fig 6 Effect of collagen on ristocetin-mediated vWF-platelet binding. ^{125}I-vWF (0.3 μg), WP (1.6 × 10^8 platelets), and ristocetin (at indicated final concentrations) were combined together in the presence of either 0.9% NaCl or collagen (175 μg/mL). Following incubation (37 °C, 30 minutes with stirring) the radioactivity of the sedimented platelet pellet was counted. Bound vWF was calculated as a percentage of maximal binding at 1.5 mg/mL. In the absence of ristocetin less than 6% binding occurred (corresponding to 9000 cpm/1.6 × 10^8 platelets), at 1.5 mg/mL maximal binding represented 160,000 cpm/1.6 × 10^8 platelets. No difference was seen in the presence of collagen.

ristocetin indicates that enhancement is not the result of nonspecific ristocetin-platelet association predisposing the platelet to an increased collagen response. Furthermore, elimination of vWF-platelet binding by chymotrypsin removal of the GPIb domain resulted in the retention of the inhibition phase and loss of the enhancement phase. With further chymotrypsin digestion, the GPIIb/IIIa domain was removed and platelets no longer aggregated with collagen, due to the removal of the fibrinogen-platelet binding site.[39]

While enhanced aggregation required a functional vWF and platelet vWF receptor, inhibition required neither and was progressively inhibited with increasing ristocetin concentration (Figs 4 and 5). The Col-PAR is therefore the summation of two independent curves: an inhibitory one due to platelet-ristocetin association, and an enhancement curve due to platelet-vWF binding. As seen in Fig 7 a decrease in platelet-vWF binding due to decreased vWF levels results in the movement of the enhancement curve to the right producing a greater inhibition eventually complete in the absence of any vWF-platelet binding. The differential response of platelet-ristocetin binding (ie, inhibition) and vWF-platelet binding (ie, enhancement) is consistent with independent membrane receptor sites for these substances [10–12]

We did find an inhibitory effect of ristocetin on the extent of collagen polymerization. This may contribute to the process of inhibition by blockage of the polymerization of soluble collagen monomer as proceeds during the lag phase

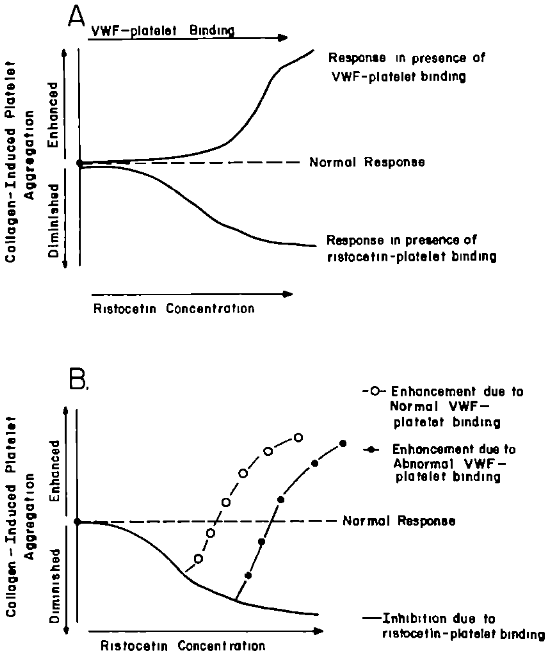

Fig 7. Interaction of ristocetin, vWF, and platelets: Effects on collagen-induced platelet aggregation as observed in the Col-PAR. (A) The normal collagen-induced platelet aggregation is diminished in the presence of ristocetin-platelet binding and enhanced in the presence of vWF-platelet binding. (B) Contribution of these two responses to the Col-PAR. In normal individuals with normal vWF-platelet binding, inhibition progresses until vWF-platelet binding is adequate to produce an enhanced response. In the presence of abnormal vWF-platelet binding (due to low vWF activity or decreased vWF-platelet binding), the enhanced response is delayed, resulting in a greater inhibition and an enhanced response outside the normal range.

of aggregation.[46,47] However, the extent of inhibition was independent of the ristocetin concentration and, furthermore, this process was not sufficient to cause inhibition in heparinized PRP, nor irreversible inhibition in citrated PRP. These findings, together with the negligent protein precipitation at the ristocetin concentrations used, also suggest that the affinity of ristocetin for plasma proteins,[48] including fibrinogen,[49] necessary for aggregation[50] is not a contributory process in inhibition. We also found an increased secondary wave of ADP-induced aggregation in the presence of vWF-platelet binding. This secondary aggregation is indicative of a platelet release reaction and similar to the release occurring during the aggregation phase of collagen-induced aggregation.[28] However, ristocetin did not inhibit nor enhance the primary ADP-platelet interaction demonstrating the unique specificity of the collagen response due to ristocetin and vWF-platelet binding. Together with previous data[51] these findings demonstrate that seemingly independent platelet-ligand interactions can influence each other.

The inhibition of collagen aggregation suggests that ristocetin interferes with an unknown specificity and mechanism with platelet sites required for adhesion and aggregation. With a critical degree of vWF-platelet binding at GPIb, however, platelet-collagen adhesion and subsequent platelet-platelet association is potentiated at a second membrane site(s). This increased in vitro adhesion is consistent with recent findings.[6-8] However in our studies the binding of vWF to the platelet, not the vWF level, was critical in the enhanced adhesion and resulted in increased interaction between platelets. This finding provides additional evidence that vWF plays a role in both platelet-subendothelial adhesion[2-5] and platelet-platelet association.[52] The potentiated adhesion may be due to a physical or electrostatic alteration of the platelet membrane by bound vWF making platelet receptor sites for collagen more readily available. There was no synergistic increase of vWF-platelet binding with ristocetin and collagen suggesting that the enhancement response is not merely an effect of quantitative vWF-platelet association. An alternative hypothesis to explain the enhanced response is that at increased levels of vWF-platelet binding, more platelets adhere to collagen through a vWF "bridging" thus causing increased aggregation.

In conclusion we have demonstrated that ristocetin inhibits in vitro collagen aggregation and the binding of vWF to platelets enhances platelet-collagen adhesion and platelet aggregation. These findings imply that, in vivo, the binding of vWF to platelets[2-5] potentiates platelet-collagen and platelet-platelet interaction upon exposure of the collagenous subendothelium at sites of vascular injury. Our studies demonstrate a role of vWF-platelet binding in the vWF-platelet-collagen interaction and demonstrate an influential interaction of ristocetin, vWF, and collagen with the platelet membrane.

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REFERENCES

1. Turitto V, Baumgartner H· Platelet-surface interactions, in Colman R (ed)· Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Philadelphia, Lippincott, 1982, p 364

2. Tschopp T, Weiss H, Baumgartner H. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. J Lab Clin Med 83.296, 1974

3. Bloom A· The von Willebrand Syndrome Semin Hematol 17.4, 1980

4. Weiss H, Baumgartner H, Tschopp T, Turitto V, Cohen D: Correction by Factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand's disease. Blood 51:267, 1978

5 Sakariassen K, Bolhuis P, Sixma J. Human blood platelet adhesion to arterial subendothelium is mediated by Factor VIII-von Willebrand factor bound to subendothelium. Nature 279:636, 1979

6. Aihara M, Cooper H, Wagner R· Platelet-collagen interactions Increase in rate of adhesion of fixed washed platelets by Factor VIII related antigen Blood 63:495, 1984

7 Houdijk W, Sakariassen K, Nievelstein P, Sixma J: Role of Factor VIII-von Willebrand Factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. J Clin Invest 75:531, 1985

8. Leytin V, Gorbunova N, Misselwetz F, Novikov I, Podrez E,

Plyusch O, Likhachova E, Repin V, Smirnov. Step by step analysis of adhesion of human platelets to a collagen coated surface. Defect in initial attachment and spreading of platelets in von Willebrand's Disease. Thromb Res 34·51, 1984

9. Hoyer L The Factor VIII complex. Structure and function. Blood 58.1, 1981

10. Jenkins C, Clemetson K, Luscher E: Studies on the mechanism of ristocetin-induced platelet agglutination: Binding of ristocetin to platelets. J Lab Clin Med 93:220, 1979

11. Coller B: The effects of ristocetin and von Willebrand factor on platelet electrophoretic mobility. J Clin Invest 61:1168, 1977

12. Coller B, Gralnick H: Studies on the mechanism of ristocetin-induced platelet agglutination—Effects of structural modification of ristocetin and vancomycin J Clin Invest 60.302, 1977

13. Moake J, Olson J, Troll J, Weinger R, Peterson D, Cimo P: Interaction of platelets, von Willebrand factor and ristocetin during platelet agglutination. J Lab Clin Med 96 168, 1980

14. Schneider-Trip M, Jenkins C, Kahle L, Sturk A, Ten Cate J: Studies on the mechanism of ristocetin-induced platelet aggregation: Binding of Factor VIII to platelets. Brit J Haemat 43:99, 1979

15 Coller B, Peerschke E, Scudder L, Sullivan C. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets· Additional evidence in support of GPIb as a platelet receptor for von Willebrand factor Blood 61:99, 1983

16. Kirby E· Factor VIII-associated platelet aggregation. Thromb Haemostas (Stuttg) 38:1054, 1977

17 Kao K-J, Pizzo S, McKee P: Platelet receptors for human Factor VIII/von Willebrand protein. Functional correlation of receptor occupancy and ristocetin-induced platelet aggregation. Proc Natl Acad Sci USA 76:5317, 1979

18. George J, Reimann T· Inherited disorders of the platelet membrane Glanzmanns Thrombasthenia and Bernard-Soulier Disease, in Colman R (ed) Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Philadelphia, Lippincott, 1982, p 496

19. Weiss H, Tschopp T, Baumgartner H, Sussman I, Johnson M, Egan J. Decreased adhesion of Giant (Bernard-Soulier) platelets to subendothelium. Further implications on the role of von Willebrand factor in hemostasis Am J Med 57 920, 1974

20. Nurden A, Dupuis D, Kunicki T, Caen J· Analysis of the glycoprotein and protein composition of Bernard-Soulier platelet by single and two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. J Clin Invest 67·1431, 1981

21. Gallop P, Seifter S. Preparation and properties of soluble collagens. Meth Enzymol 6.635, 1963

22. Kessler C, Floyd C, Rick M, Krizek D, Lee S, Gralnick H. Collagen-Factor VIII/von Willebrand factor protein interaction. Blood 63:1291, 1984

23. Ts'ao C, Lo R, Raymond J: Critical importance of citrate-blood ratio in platelet aggregation studies. Am J Clin Path 65:518, 1976

24. Born G: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927, 1962

25. Coller B, Franza B, Gralnick H. The pH dependence of quantitative ristocetin-induced platelet aggregation. Theoretical and practical implications—A new device for maintenance of platelet-rich plasma pH. Blood 47 841, 1976

26. Mastacchi R, Stanzani L, Barbanti M, Montecchi L, Bianchini P· Interaction of heparin and heparin fractions with human platelets Thromb Res 28.275, 1982

27. Pekcelen Y, Inceman S: Heparin and ristocetin-induced platelet aggregation Thromb Haemostas (Stuttg) 35:485, 1976

28 Marcus AJ: Platelet aggregation, in Colman R (ed): Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Philadelphia, Lippincott, 1982, p 380

29. Robson E, LaDuca F, Bettigole R, Small B: Quantitation of platelet aggregation using adenosine diphosphate Blood 62:5, 1983 (abstr)

30. Robson E, LaDuca F, Small B, Bettigole R: Quantitation of platelet aggregation using collagen. Blood 65:251a, 1984 (abstr)

31. Draper N, Smith H: Applied regression analysis (2nd ed). New York, Wiley, 1981, p 241

32. Zimmerman T, Hoyer L, Dickson L, Edgington T: Determination of the von Willebrand's Disease antigen (factor VIII–related antigen) in plasma by quantitative immunoelectrophoresis. J Lab Clin Med 86:152, 1975

33. Frojmovic M, Milton J, Duchastel A: Microscopic measurements of platelet aggregation reveal a low ADP-dependent process distinct from turbidimetrically measured aggregation. J Lab Clin Med 101:964, 1983

34. Reuter H, Deters K: Comparative investigation on the quantitative determination of ADP and adrenalin-induced aggregation by means of a microscopic test and an electronic platelet counting method. Thromb Res 15:711, 1979

35. Lowry O, Rosebrough N, Farr A, Randall R· Protein measurement with the folin phenol reagent. J Biol Chem 193:265, 1951

36. Jenkins C, Meyer D, Dreyfus M, Larrieu M: Willebrand factor and ristocetin I. Mechanism of ristocetin-induced platelet aggregation Brit J Haemat 28:561, 1974

37. Walsh P· Albumin density gradient separation and washing of platelets and the study of platelet coagulant activities. Brit J Haemat 22:205, 1972

38 Greenberg J, Packham M, Guccione M, Harfenist E, Orr J, Kinlough-Rathbone R, Perry D, Mustard J: The effect of pretreatment of human or rabbit platelets with chymotrypsin and their responses to human fibrinogen and aggregating agents. Blood 54:753, 1979

39. Peerschke E, Coller B· A murine monoclonal antibody that blocks fibrinogen binding to normal platelets also inhibits fibrinogen interactions with chymotrypsin treated platelets Blood 64.59, 1984

40. Fairbanks G, Steck T, Wallach D. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606, 1971

41. Rosse W, Devine D, Ware R: Reactions of immunoglobulin G-binding ligands with platelets and platelet-associated immunoglobulin G. J Clin Invest 73:489, 1984

42. Simone J, Vanderherder J, Albildgaard C· A semiautomatic one stage Factor VIII assay with a commercially prepared standard. J Lab Clin Med 69:706, 1967

43. Allain J, Cooper H, Wagner R, Brinkhous K: Platelets fixed with paraformaldehyde: A new reagent for assay of von Willebrand factor and platelet aggregating factor. J Lab Clin Med 85·318, 1975

44 Kasper C, Aledort L, Counts R, Edson R, Frantantoni J, Green D, Hampton J, Hilgartner M, Lazerson J, Leven P, McMillin C, Poon J, Shapiro S, Shulman N, Von Eys J: A more uniform measurement of Factor VIII inhibitors. Thromb Diathes Haemorrh 34:869, 1975

45. Phillips D, Agin A: Platelet membrane glycoproteins: Identification of a proteolytic substrate for thrombin. Biochem Biophys Res Commun 75:940, 1977

46. Jaffe R, Deykin P: Evidence for structural requirement for the aggregation of platelets by collagen. J Clin Invest 55:875, 1974

47 Gordon J: Mechanism of platelet-collagen interaction. Nature 278.13, 1979

48. Baugh R, Brown J, Hougie C· Plasma components which interfere with ristocetin-induced platelet aggregation. Thrombos Diathes Haemorrh (Stuttg) 33.540, 1975

49 Suzuki K, Nishioka J, Hashimoto S. A simple technique for

purification of fibrinogen from plasma by affinity chromatography on ristocetin-agarose. Thromb Res 18:707, 1980

50. Mustard J, Packham M, Kinlough-Rathbone R, Perry D, Ragoeczi E. Fibrinogen and ADP-induced platelet aggregation. Blood 52:453, 1978

51. Grant R, Zucker M, McPherson J: ADP-induced inhibition of von Willebrand factor mediated platelet agglutination. Am J Physiol 230:1406, 1976

52. Turitto V, Weiss H, Baumgartner H. Platelet interaction with rabbit subendothelium in von Willebrand's disease: Altered thrombus formation distinct from defective platelet adhesion. J Clin Invest 74:1730, 1984

Cytotoxicity of Interleukin 2–Activated Lymphocytes for Leukemia and Lymphoma Cells

By Kazuo Oshimi, Yoko Oshimi, Miyuki Akutsu, Yayoi Takei, Hiroshi Saito, Michiko Okada, and Hideaki Mizoguchi

Studies were undertaken to determine whether leukemia and lymphoma cells would be lysed by autologous and allogeneic lymphokine-activated killer (LAK) cells. Peripheral blood mononuclear cells (PBMC) from patients and normal donors were cultured for five days, 2 weeks, and 4 weeks with medium containing 2,500 units of recombinant interleukin 2 (IL-2) per mL, and their cytotoxicity was assayed by a five-hour ^{51}Cr-release test. Of primary tumors isolated from patients with acute nonlymphoblastic leukemia, acute lymphoblastic leukemia, and non-Hodgkin's lymphoma, tumors of 37 out of 40 patients tested were shown to be susceptible to normal donors' LAK, and tumors of 18 of 20 patients tested were shown to be susceptible to autologous LAK. LAK cultured for longer periods showed a tendency to have lower cytotoxicity. LAK had also low, but significant, levels of cytotoxicity for nonmalignant target cells. Because PBMC expanded in IL-2–containing medium consisted mainly of OKT3-positive pan T cells, OKT8-positive suppressor/cytotoxic cells, and Leu-11–positive natural killer (NK) cells, and treatment with OKT3 and Leu-11 monoclonal antibodies (mAb) reduced LAK activity for autologous and allogeneic tumor cells, both T and NK cells appeared to be effector cells for LAK activity. Mechanisms of target-cell recognition in the LAK system seem to be different from those in alloreactive cytotoxic T lymphocytes (CTL) based on the results that, while cytotoxicity of alloreactive CTL was inhibited by the treatment of effector cells with mAb, OKT3, and OKT8, and by the treatment of target cells with a mAb that reacts with HLA class I antigen, LAK activity was not inhibited by the above treatment. When chromosomes of IL-2–expanded PBMC in nine patients and two normal individuals were analyzed, PBMC from one patient showed chromosomes of clonal abnormalities, and PBMC from five donors showed those of nonclonal abnormalities.

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INTERLEUKIN 2 (IL-2) is a lymphokine produced by helper T cells that stimulates the growth of activated murine and human lymphocytes in vitro.[1,2] The incubation of normal lymphoid cells with IL-2 leads to the generation of lymphokine-activated killer (LAK) cells that can lyse fresh tumor cell targets in short-term ^{51}Cr-release assay.[3–7] It has been shown that the adoptive transfer of these in vitro–derived LAK cells can mediate the regression of established metastasis from murine tumors.[8–11]

The antitumor effect of LAK has prompted attempts to elucidate the spectra of human tumor cells sensitive to LAK, and it has been shown that various types of sarcoma, melanoma, and cancers of the colon, ovary, pancreas, esophagus, and adrenal glands are lysed by autologous or allogeneic LAK effector cells.[3,4,12] Of hematological malignancies, acute lymphoblastic leukemia (ALL) cells derived from a patient were shown to be insensitive to autologous LAK,[3] and lymphoma cells derived from three patients were shown to be sensitive to allogeneic LAK.[12] There have been no positive reports, however, as to whether hematologic malignant cells isolated from patients are sensitive to autologous LAK, nor has it been determined which subtypes of leukemia or lymphoma cells are sensitive to LAK.

The experiments reported in this article were initiated with three aims. First, considering the potential application of LAK in clinical trials, it was determined whether leukemia and lymphoma cells isolated from patients would be lysed by autologous and allogeneic short-term (five-day) cultured LAK. Second, in view of the fact that a more potent antitumor effect was demonstrated when a larger number of LAK cells was inoculated into experimental animals,[10] we tested whether in vitro–expanded LAK in a longer period (2 to 4 weeks) of culture in IL-2–containing medium would also lyse autologous and allogeneic tumor cells. Third, if LAK is found to be lytic for these targets, its adverse effects must be considered. Therefore we tested whether LAK also lyses nonmalignant target cells and whether peripheral blood mononuclear cells (PBMC) expanded in vitro with IL-2–containing medium develop chromosomal abnormalities. The results show that short-term and longer term cultured LAK lysed autologous and allogeneic leukemia and lymphoma cells of most of the patients tested. However, LAK also had a low, but significant, level of cytotoxicity for autologous and allogeneic nonmalignant target cells, and chromosomal changes of a clonal nature were demonstrated in in vitro–expanded PBMC.

MATERIALS AND METHODS

Characteristics of patients and tumor cells. Patients with acute nonlymphoblastic leukemia (ANLL), ALL, and non-Hodgkin's lymphoma were studied The diagnosis of leukemia was based on the French-American-British (FAB) cooperative group designation,[13] and that of lymphoma was based on the working formulation of the National Cancer Institute.[14] Patients with more than 80% malignant cells in their peripheral blood leukocytes, bone marrow cells, or lymph node cells were selected as donors of tumor cells. The percentage of tumor cells in the specimens was determined from morphology and surface markers. When bone marrow cells were obtained from patients and normal volunteers, the donors were advised of the procedure and attendant risks and gave informed consent These studies were approved by the Human Experimentation Committee of our college.

Surface marker analysis Leukemic cells were separated from the peripheral blood or bone marrow cells by Ficoll-Conray gradient centrifugation, and lymphoma cells were obtained by teasing lymph nodes with forceps. The specimens were assayed for their surface phenotypes by the indirect immunofluorescence method as described elsewhere.[15,16]

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Surface markers of IL-2–expanded PBMC were analyzed by flow cytofluorometry (Spectrum III; Ortho Diagnostic Systems, Westwood, Mass) Monoclonal antibodies (mAb) used were OKT3 (pan T), OKT4 (helper/inducer T), OKT8 (suppressor/cytotoxic T), anti-B_1 (pan B),[17] and Leu-11a which reacts with natural killer (NK) cells.[18] For the direct staining of T, B, and NK antigens, 1 to 2 × 10^6 cells were incubated with saturating concentrations of fluorescein isothiocyanate (FITC)–conjugated mAb for 30 minutes at 4 °C, and assayed after being washed twice with phosphate-buffered saline For two-color immunofluorescence analysis using OKT3 and Leu-11a mAb, 1 to 2 × 10^6 cells were incubated with 10 μL of OKT3 at 4 °C for 30 minutes, washed once thereafter, and 100 μL of 1:20-diluted biotinyl goat antimouse immunoglobulin (Ig) antibody (Becton Dickinson Monoclonal Center Inc, Mountain View, Calif) was added After being incubated for 30 minutes at 4 °C, the cells were washed once and exposed to 100 μg of mouse IgG (Cappel Laboratories, West Chester, Pa) for ten minutes at 4 °C to block goat antimouse Ig antibody activity. Then 100 μL of 1.20-diluted phycoerythrin-conjugated avidin (Becton Dickinson) and 5 μL of FITC-conjugated Leu-11a mAb were added, and the cells were incubated for 30 minutes at 4 °C. The cells were washed once and passed on Spectrum III by using an argon ion laser at 488 nm excitation wavelength. Green fluorescence (515 to 530 nm wavelength) from FITC, and red (>530 nm) from phycoerythrin were detected independently and displayed as dot plots in the linear scale of fluorescence intensity

Induction of LAK Heparinized whole blood was obtained from patients and two normal donors. Patients' blood was taken at the period when leukemia or lymphoma cells were not present in the peripheral blood. Because it is not known whether chemotherapy or radiotherapy affects LAK precursors, blood was taken before the initiation of therapy or at least 3 weeks after the cessation of therapy. PBMC were separated with Ficoll-Conray density gradients, washed twice, and suspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (Flow Laboratories, McLean, Va), 2,500 units of recombinant IL-2 per mL, 2 mmol/L L-glutamine, 100 units of penicillin per mL, and 10 μg of streptomycin per mL. Initially 2 × 10^6 PBMC were cultured in a 24-well tissue culture plate (Linbro Division, Flow Laboratories, Hamden, Conn) in 2 mL of the above complete medium at 37 °C in humidified air containing 5% CO_2. After five days of culture, cells were assayed for proliferative response and LAK activity For the assay of proliferative response, 0.15 mL quantities of the cultured cell suspension were transferred to the wells of a microculture plate in triplicate and 1 μCi of ^{3}H-thymidine was added to each well. After 18 hours of further incubation at 37 °C, the cells were harvested on glass fiber filters and the incorporation of radioactivity was measured by a liquid scintillation counter. For the assay of LAK activity, cultured PBMC were washed once and used as effector cells for the cytotoxicity assay as will be described later. When PMBC were propagated with IL-2–containing medium, the complete medium was changed every three or four days and the concentration of cells was maintained in the range of 5 × 10^5/mL to 3 × 10^6/mL. Cells from the two normal donors proliferated 30- to 100-fold during the first 2 weeks of culture and 10- to 20-fold during the second 2 weeks of culture. After 2 and 4 weeks of culture, cells were harvested and assayed for their LAK activity.

IL-2 used for the induction of LAK activity was produced by recombinant DNA technology,[19] and was provided by Shionogi Pharmaceutical Co Ltd, Osaka, Japan. The purity was more than 99%, and the specific activity of this IL-2 was 1 2 × 10^7 units/mg of protein. IL-2 activity was assayed by the method described by Gillis et al,[20] and the units were determined on the basis of a reference Jurkat IL-2 standard provided by the Biological Response Modifiers Program of the National Cancer Institute.

Assay for LAK activity A five-hour ^{51}Cr-release test[21] was used for the assay of LAK activity. Target cells were leukemia and lymphoma cells isolated from the patients, an NK-sensitive cell line K562, and an NK-insensitive cell line Raji. Freshly isolated tumor cells were suspended in Eagle's minimum essential medium containing 75% fetal calf serum (FCS) and 10% dimethylsulfoxide, and stored in liquid nitrogen When used as targets in the cytotoxicity assay, they were thawed and centrifuged with Ficoll-Conray to remove dead cells. Cells of more than 70% viability were used as targets The susceptibility to five-day and 4-week cultured LAK of frozen K562 cells, Raji cells, and PBMC from normal individuals used immediately after thawing did not differ from that of K562 and Raji cells in culture and of PBMC freshly isolated from the same individuals. For the labeling of target cells in the cytotoxicity assay, 50 μCi or 300 to 400 μCi of $Na_2{}^{51}CrO_4$ (Japan Isotope Association, Tokyo, Japan) was incubated with 10^6 pelleted cells for two hours Fifty microcuries were used for the labeling of cultured cell lines, and 300 to 400 μCi for the leukemia and lymphoma cells isolated from the patients. The labeled cells were then washed three times, and used as targets at 8 × 10^3 cells per microculture well. Effector-to-target (E T) ratios were 80:1 and 20:1 when patients' malignant cells were used as targets, and 20:1 and 5.1 when cultured cell lines were used as targets. The experiments were performed in triplicate. After five hours of incubation, the supernatant fluid was harvested and the radioactivity was determined with a gamma well counter. The specific ^{51}Cr release was calculated by the following formula: % release = [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100, where spontaneous release was the ^{51}Cr release from 8 × 10^3 labeled target cells incubated alone in the medium, and maximum release was that from labeled target cells incubated alone in water containing 5% detergent The results of cytotoxicity tests were expressed as mean percent specific ^{51}Cr release. The standard deviation of percent specific ^{51}Cr release was less than 5% in most of the experiments. Cytotoxic activity was considered positive when the value of experimental ^{51}Cr release was significantly above that of spontaneous ^{51}Cr release according to the Student's *t* test.

Induction of anomalous killer activity and lectin-dependent cellular cytotoxicity Because LAK efficiently lyses patients' tumor cells, as will be presented in Results, the level of LAK activity was compared in the same individual with that of known nonspecific killer cell activity of anomalous killer (AK)[22,23] and lectin-dependent cellular cytotoxicity (LDCC).[24] Because cells mediating AK activity are induced following mixed lymphocyte culture (MLC), PBMC of a normal donor were cultured for five days with the same number of mitomycin C-treated allogeneic PBMC, and then assayed for their cytotoxicity against leukemia and lymphoma cells isolated from the patients. Preliminary experiments revealed that the highest level of AK activity was induced after five days of MLC. For the assay of LDCC, PBMC freshly prepared from the donor were incubated with ^{51}Cr-labeled target cells in the presence of 1·3,000 final concentration of phytohemagglutinin-P (PHA-P) (Gibco Laboratories, Grand Island, NY). The 1 3,000 final concentration was selected because in preliminary experiments the highest level of LDCC was obtained at that concentration of PHA-P. Cytotoxicity assay used for AK and LDCC was the five-hour ^{51}Cr-release test with E T ratios of 80.1 and 20:1, as described above.

Characterization of LAK effector cells. The panning method described previously[21] was modified as follows. Three-milliliter quantities of affinity-purified F(ab')$_2$ fragment goat antimouse IgG or IgM (Cappel Laboratories) were added to 60 mm Petri dishes. After overnight incubation at 4 °C, the antibody was harvested from the plates and each plate was washed three times with 3 mL of cold medium. Cultured PBMC, 8 × 10^6, resuspended in 0.5 mL of 1:10-diluted OKT3 or Leu-11b mAb were incubated on ice for 30 minutes, washed, resuspended in 2.5 mL of RPMI 1640 medium containing 5% FCS, and added to the antibody-coated plates. The plates were then incubated in a 5% CO_2 incubator for 45 minutes at 37 °C. The nonadherent cells were harvested by swirling the plates and removing the cells by aspiration with a Pasteur pipette. In preliminary experiments more than 95% of the $OKT3^+$ or Leu-$11b^+$ cells were removed by the panning method when the cells were assayed by the indirect immunofluorescence method. The nonadherent cells were then centrifuged, and the pellet was resuspended in 1 mL of RPMI 1640 medium containing 10% FCS. The percentage of cells yielded was determined, and 0.1 mL quantities of the cells were transferred to wells of a microculture plate in triplicate. Therefore, if the yield of the cells was 100%, the E:T ratio in the cytotoxicity assay should be 80:1.

Analysis of recognition mechanisms in LAK-mediated target cell lysis with mAb. The nature of receptors on LAK that recognize antigenic structures on target cells is not known, nor is that of antigenic structures on target cells. Therefore, in order to test whether similar mechanisms of antigen recognition are present in the LAK system when compared with alloreactive cytotoxic T lymphocytes (CTL), LAK effector cells were preincubated with mAb OKT3, OKT4, or OKT8 for 30 minutes at 37 °C prior to the addition of ^{51}Cr-labeled target cells, and, in a reciprocal fashion, ^{51}Cr-labeled target cells were preincubated with mAb W6/32[25] (Sera Laboratories, Sussex, England), which reacts with a nonpolymorphic determinant on the α chain of HLA-A and -B, or with OKIa1[26] which reacts with P23, 30 compartments of Ia antigen prior to the addition of LAK effector cells. At the same time, seven-day MLC-induced alloreactive CTL and PHA-induced target lymphocytes were preincubated with mAb in the same manner.

Chromosome analysis. Chromosomes were examined in marrow aspirates obtained from the patients and in IL-2–expanded PBMC from the patients and two normal individuals. Chromosome slides were prepared by the 24-hour cultivation technique. A synchronization method using methotrexate and thymidine was also utilized for Q-banding analysis.[27] The chromosome number was determined in 30 to 40 cells, and detailed analysis was performed in 5 to 10 cells. An abnormal clone was defined as two cells with the same extra chromosome or structural rearrangement, or three cells with the same missing chromosome, according to the criteria of the First International Workshop on Chromosomes in Leukemia.[28]

RESULTS

Optimal conditions for LAK development and cellular proliferation. For induction of LAK activity in short-term cultured PBMC, the optimal concentration of IL-2 was determined. PBMC from a normal donor were incubated with various concentrations of IL-2 for five days, and assayed for their cytotoxicity for leukemia and lymphoma cells isolated from patients. Although details are not shown, the concentrations of IL-2 of 250–50,000 U/mL showed almost the same maximum level of LAK activity for leukemia and lymphoma cells. Raji and K562 cell lines were also efficiently lysed by the same concentrations of IL-2. Next, the kinetics of the development of LAK lytic activity were studied. When PBMC from a normal donor were cultured with 2,500 units of IL-2 per mL for various lengths of time, the level of highest cytotoxicity was obtained after five days of culture (Fig 1). Therefore a culture period of five days was selected for the following experiments for the activation of LAK in short-term culture.

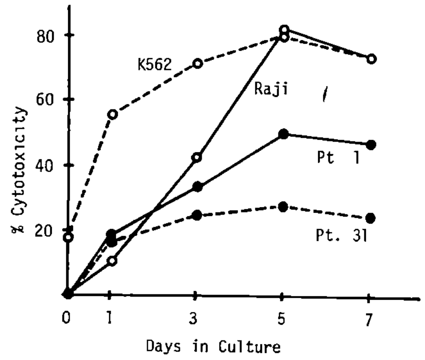

Fig 1. Kinetics of LAK development. PBMC from a normal donor were cultured for various lengths of time in medium containing 2,500 units of IL-2 per mL, and their cytotoxicity was assayed against ANLL cells from patient 1, lymphoma cells from patient 31, and cell lines K562 and Raji. In the cytotoxicity assay, the E:T ratio was 80:1 for ANLL and lymphoma cells, and 20:1 for cell lines.

Because experiments on in vitro expansion of PBMC in longer term culture with medium containing IL-2 were attempted in the present study, the optimal concentration of IL-2 for cellular proliferation was determined. PBMC from a normal donor were incubated with various concentrations of IL-2 for five days, and assayed for their proliferative response by ^{3}H-thymidine incorporation. Concentrations of IL-2 of 500 to 5,000 U/mL were most effective for the proliferation of PBMC (data not shown). These findings, taken together, led to the selection of a concentration of 2,500 units of IL-2 per mL for the following experiments on both induction of LAK activity in short-term culture and expansion of PBMC in longer term culture.

LAK activity of normal donors against leukemia and lymphoma cells isolated from patients. PBMC from two normal donors were cultured for five days, 2 weeks, and 4 weeks, and their LAK activity was assayed against leukemia and lymphoma cells isolated from the patients (Figs 2, 3, 4). Of ANLL blast cells obtained from 18 patients, those of 17 patients were sensitive to LAK of either donor I or donor II, or both (Fig 2). Monoblasts obtained from patient 16 were LAK-resistant leukemic cells, whereas monoblasts of the other two patients, 17 and 18, were sensitive. Blast cells from all nine patients with ALL were sensitive (Fig 3). Of lymphoma cells from 13 patients, those of 11 patients were sensitive (Fig 4). Of the ALL patients, patients 19 and 21 had T-cell ALL, patient 22 had non-T, non-B ALL, and the others had ALL of B-cell lineage. Of lymphoma patients, patients 30, 39, and 40 had T-cell lymphoma, and the others had B-cell lymphoma. Thus susceptibility to LAK was not correlated with T or B surface phenotypes. According to Nadler et al,[17] ALL cells in the most primitive stage of B-cell differentiation bear B_4 antigen, and, with maturation, they acquire common ALL antigen, and then B_1 antigen. ALL cells of patients 23, 24, and 25 had B_4 antigen alone, those of patients 20 and 26 had B_4 and common ALL antigens, and those of patient 27 had B_4, common ALL, and B_1 antigens. When susceptibility was compared among ALL cells in different stages, those in the most primitive stage were shown to be more susceptible than those in more mature differentia-

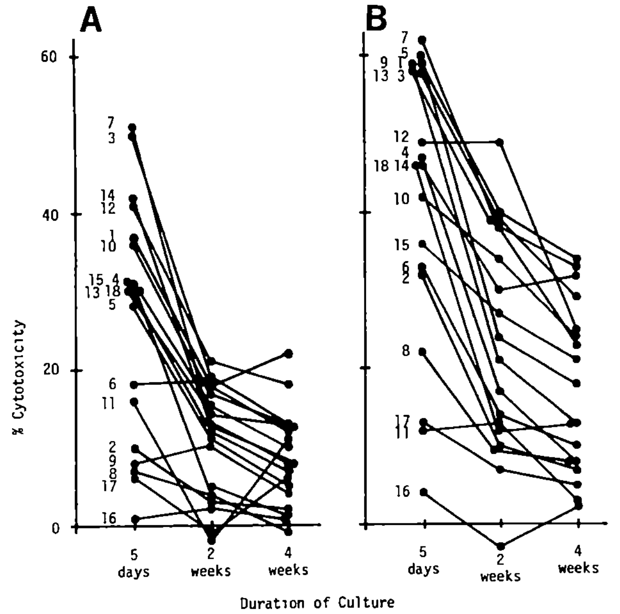

Fig 2. LAK activity of normal donors' PBMC against ANLL cells isolated from patients. PBMC from normal donors I (A) and II (B) were cultured for five days, 2 weeks, and 4 weeks in the presence of 2,500 units of IL-2 per mL, and their cytotoxicity was assayed against ANLL cells isolated from patients by a five-hour ^{51}Cr-release test. The percentage of cytotoxicity recorded was that of the higher level of cytotoxicity at an E:T ratio of either 80:1 or 20:1, and in most of the experiments, cytotoxicity was higher when the E:T ratio was 80:1. Numbers in the figure represent patient number. Patients 1 through 4 were diagnosed as having M1 ANLL, 5 through 7 were M2, 8 through 10 were M3, 11 through 15 were M4, and 16 through 18 were M5.

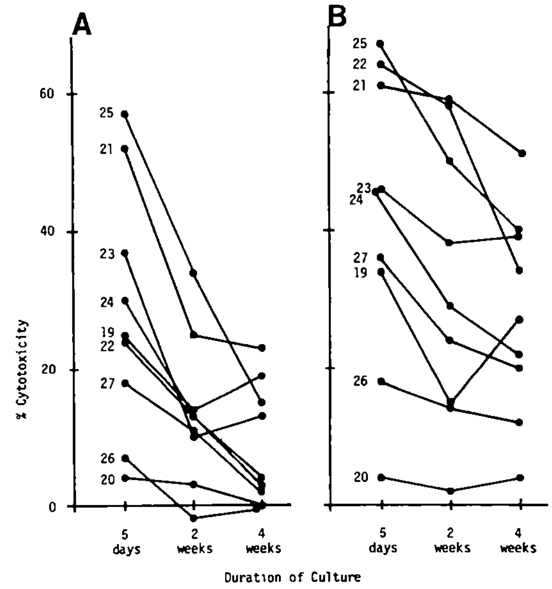

Fig 3. LAK activity of normal donors' PBMC against ALL cells isolated from patients. PBMC from normal donors I (A) and II (B) were treated as described in the legend of Fig 2. Patients 19 and 20 had L1 ALL and patients 21 through 27 had L2 ALL.

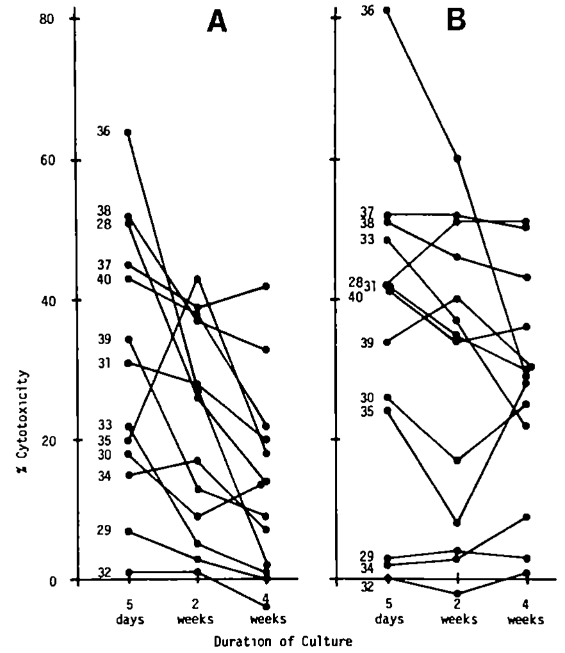

Fig 4. LAK activity of normal donors' PBMC against lymphoma cells isolated from patients with non-Hodgkin's lymphoma. PBMC from normal donors I (A) and II (B) were treated as described in the legend of Fig 2. Patient 28 had follicular, small cleaved cell lymphoma, patient 29 had diffuse, small cleaved cell lymphoma, patients 30 through 35 had diffuse, large cell lymphoma, patients 36 and 37 had large cell, immunoblastic lymphoma, patient 38 had Burkitt's lymphoma, and patients 39 and 40 had lymphoblastic lymphoma.

tion stages (in donor I, $P < 0.05$ for five-day and 4-week cultured LAK, and in donor II, $P < 0.05$ for all LAK cells). It is worth noting that lymphoma cells of clinically high-grade malignancy[14] (patients 36 to 40) were also more susceptible than those of low-grade and intermediate-grade malignancy[14] (patients 28 to 35) (in donor I, $P < 0.01$ for five-day LAK; in donor II, $P < 0.05$ for five-day and 2-week LAK). Cytotoxicity of five-day cultured LAK was significantly higher than that of 2-week cultured LAK ($P < 0.0001$ in donor I and $P < 0.00001$ in donor II), and that of 2-week cultured LAK was significantly higher than that of 4-week cultured LAK ($P < 0.001$ in donors I and II). LAK activity of donor II was significantly higher than that of donor I in five-day, 2-week, and 4-week culture ($P < 0.00001$). Tumors of patients 2 and 9 were poorly lysed by donor I but well lysed by donor II, with tumors of patients 11, 28, and 34 better lysed by donor I. This tendency, however, became less striking in repeated experiments (data not shown). When PBMC from the normal donors were cultured for five days in the absence of IL-2, cytotoxicity was not demonstrated in any of the experiments against tumor cells of patients 1, 2, 5, 8, 20, 25, 30, 31, 36, and 49 (data not shown).

LAK activity against autologous tumor cells. As shown in Fig 5, a significant level of cytotoxicity for autologous leukemia or lymphoma cells was demonstrated in PBMC of 17 of 20 patients after five days of culture, in those of 18 of 19 patients after 2 weeks of culture, and in those of 15 of 19 patients after 4 weeks of culture. In repeated experiments,

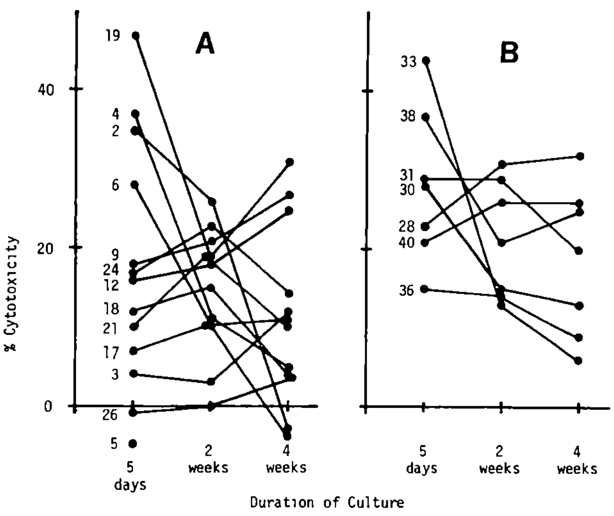

Fig 5. LAK activity against autologous tumor cells. PBMC from the patients were cultured for five days, 2 weeks, and 4 weeks as described in the legend of Fig 2, and their cytotoxicity was assayed against autologous ANLL or ALL cells (A) and lymphoma cells (B).

patients 5 and 26 again demonstrated no cytotoxicity against their autologous tumors, although the tumors were lysed by allogeneic normal donors' LAK. What was responsible for this lack of lysis was not determined. The level of cytotoxicity of 4-week cultured LAK at an E:T ratio of 80:1 was significantly lower than that of five-day cultured LAK ($P < 0.05$). When PBMC were cultured for five days in the absence of IL-2, cytotoxicity for autologous tumor cells was not demonstrated in any of the experiments in patients 2, 9, 24, 31, 38, and 40 (data not shown).

Cytotoxicity for Raji and K562 cells was also assayed each time LAK activity for autologous tumors was assayed, and the level of cytotoxicity was compared between autologous tumor cells versus Raji cells, and between autologous tumor cells versus K562 cells. As shown in Fig 6, significant correlation was demonstrated ($P < 0.00001$ in autologous cells versus Raji cells, and $P < 0.001$ in autologous cells versus K562 cells). Thus, the level of LAK activity for autologous tumor cells was roughly parallel to that of LAK activity for Raji and K562 cells.

Comparison of LAK activity with AK activity and LDCC. Because normal donors' LAK lysed tumor cells of more than 90% of patients, LAK activity of a normal donor was compared with AK activity and LDCC from the same individual against tumor cells isolated from 10 patients. Fig 7 shows that the level of LAK activity was much higher than that of AK and LDCC. When freshly prepared PBMC were assayed without the addition of PHA, cytotoxicity was not demonstrated or was lower, if present at all, than that of LDCC (data not shown).

LAK activity against nonmalignant target cells. LAK activity of normal donors I and II and some patients was assayed against autologous and allogeneic nonmalignant target cells Target cells used were normal PBMC, normal bone marrow cells, and nonmalignant lymph node cells obtained from patients with reactive lymphadenitis. The results shown in Table 1 demonstrate that LAK had a low, but significant, level of cytotoxicity for autologous and allogeneic nonmalignant target cells in most of the experiments. When PBMC from the two normal donors were cultured for five days in the absence of IL-2, cytotoxicity for autologous and allogeneic PBMC and for allogeneic lymph node cells was not demonstrated (data not shown).

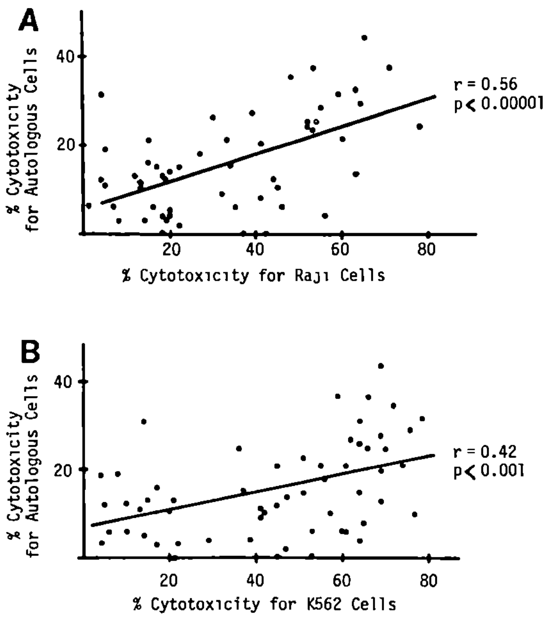

Fig 6. Correlation of cytotoxicity for autologous tumor cells and cell lines. Five-day, 2-week, and 4-week cultured PBMC from the patients were assayed for their cytotoxicity against autologous tumor cells and cell lines Raji and K562 cells. (A) E:T ratios in the cytotoxicity assay were 80:1 for autologous tumor cells and 20:1 for Raji cells. (B) The E:T ratios were 80:1 for autologous tumor cells and 5:1 for K562 cells. When the percent cytotoxicity was below zero, it was plotted as zero in the figures and treated as zero in the statistical analyses. When an E:T ratio of 20:1 was used instead of 5:1 in the cytotoxicity assay for K562 cells, the correlation coefficient r was 0.38 and P was less than 0.005.

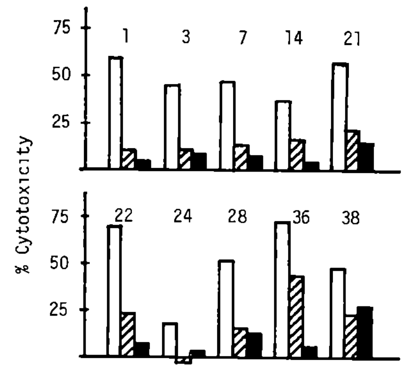

Fig 7. Comparison of LAK activity with AK and LDCC. The activity of five-day cultured LAK from a normal donor was compared with that of AK and LDCC. AK was induced after five days of MLC, and LDCC was assayed with PBMC freshly prepared in the presence of a 1:3,000 dilution of PHA-P. Target tumor cells were those isolated from 10 patients (number in the figure indicates patient number), and the E:T ratio in the cytotoxicity asssay was 80:1. LAK(□), AK(▨), LDCC(■).

Table 1. LAK Activity Against Nonmalignant Target Cells

Target Cells*		Percentage of Cytotoxicity					
		PBMC of Donor I			PBMC of Donor II		
		Cultured			Cultured		
		5 d	2 wk	4 wk	5 d	2 wk	4 wk
PBMC of donor I	exp 1	12†	15†	6†	25†	11†	7†
	2	3	1	0	2	3	1
	3	10†	ND‡	ND	12†	ND	ND
PBMC of donor II	exp. 1	8†	6†	0	21†	3	2
	2	2	0	−2	2	1	2
	3	2	ND	ND	7†	ND	ND
Bone marrow cells	normal	22†	2	3	13†	7†	12†
	Pt 9	6†	5†	1	23†	9†	3†
Lymph node cells	Pt 41	12†	3	4	10†	15†	14†
	Pt. 42	14†	4	4	17†	9†	11†
	Pt 43	9†	8†	7†	18†	14†	10†
PBMC	Pt. 24§	8†	4†	1			
Bone marrow cells	Pt. 9§	10†	−6	3			
	Pt. 17§	ND	7†	ND			
Lymph node cells	Pt. 43§	14†	9†	10†			

PBMC from normal donors I and II and from patients were cultured for five days, 2 weeks, and 4 weeks in medium containing 2,500 units of IL-2 per mL, and their cytotoxicity was assayed against nonmalignant target cells. The percent cytotoxicity recorded was that of the higher level of cytotoxicity at an E:T ratio of either 80:1 or 20:1, and in most of the experiments, cytotoxicity was higher when the E:T ratio was 80:1.

*Target cells were freshly isolated PBMC from normal donors I and II and from patient 24, bone marrow cells from a normal donor and from patients 9 and 17 with leukemia in complete remission, and lymph node cells from three patients, 41, 42, and 43, with reactive lymphadenitis.

†Percentage of cytotoxicity in a ^{51}Cr-release assay in which the value of released ^{51}Cr was significantly ($P < 0.05$) higher than that of spontaneously released ^{51}Cr.

‡Not done.

§LAK assay in the autologous effector-target systems.

Surface marker analysis of in vitro–expanded PBMC PBMC from the two normal donors were expanded in vitro with IL-2–containing medium, and their surface markers were examined after 2 to 4 weeks of culture. The results shown in Table 2 indicate that expanded cells were mainly T3 positive, T8 positive, and Leu-11a positive. In contrast, T4-positive cells were less expanded, and B_1-positive cells were not expanded. Two-color fluorescence analysis revealed that there were only a few cells simultaneously expressing T3 and Leu-11a antigens.

Characterization of LAK effector cells. Because the proliferating cells were T3-positive cells and Leu-11–positive cells as shown in Table 2, and because conflicting results were reported on the nature of LAK effector cells, ie, Rosenberg's group reported that they are T cells,[4,29,30] Itoh et al[31] reported that they are NK cells, and Nishimura and Hashimoto,[32] and Merluzzi[33] reported that they are both T cells and NK cells, we studied the surface markers of LAK effector cells by negative selection with mAb (Table 3). The results indicate that both T3-positive T cells and Leu-11–positive NK cells are effector cells.

Analysis of recognition mechanisms in LAK-mediated target cell lysis with mAb. Table 4 demonstrates that preincubation of CTL with mAb OKT3 and OKT8 inhibited alloreactive CTL activity. CTL cytotoxicity was also inhibited by preincubation of target cells with mAb W6/32, which reacts with HLA class I antigen. In contrast, LAK activity for patients' tumor cells or autologous PBMC was not inhibited by mAb treatment, suggesting that target cell recognition mechanisms in the LAK system are different from alloreactive CTL.

Chromosome analysis of in vitro–expanded PBMC. Chromosomes of PBMC were analyzed after in vitro culture in nine patients in whom a prior chromosome study was performed in bone marrow cells, and in the two normal individuals As shown in Table 5, in PBMC from five donors or in six out of 15 samples, nonclonal abnormalities were found, and in patient 6, six out of 10 cells showed clonal proliferation of abnormal cells having the chromosome of 45, X, −X, t(6;8), 15q−. Because clonotypic abnormalities developed only in a single patient, this finding should not be generalized to other patients.

DISCUSSION

Although clinical application of IL-2–expanded bulk (nonclonal) T cells or T-cell clones cytotoxic for autologous tumor cells has been greatly anticipated, it has been hampered by difficulty in inducing and expanding T cells with specific killer activity.[34-37] In the NK system, NK cells having lytic activity for autologous tumor cells have been described,[38-40] yet these NK cells were also cytotoxic for autologous normal blood mononuclear cells,[21] and NK cells lost their ability to lyse autologous tumor cells in most of the experiments after expansion of NK in IL-2–containing

Table 2. Surface Marker Analysis of Fresh and IL-2–Expanded PBMC of Normal Donors

mAb	Percentage of Positive Cells					
	Donor I's PBMC			Donor II's PBMC		
	Fresh	2 wk Cultured	4 wk Cultured	Fresh	2 wk Cultured	4 wk Cultured
OKT3	75	81 ± 4 (3)	85 ± 5 (4)	63	58 ± 4 (3)	62 ± 5 (4)
OKT4	59	16	12 ± 5 (3)	41	2	1 ± 0 (3)
OKT8	19	64	72 ± 5 (3)	24	43	51 ± 2 (3)
Leu-11a	14	21	23 ± 3 (2)	24	29	39 ± 14 (2)
anti-B_1	5	0 ± 0 (2)	0 ± 0 (2)	9	0 ± 0 (2)	0 ± 0 (2)
OKT3 & Leu-11a	1*	1*	4*	0*	0*	2*

Fresh and IL-2-expanded PBMC from the two normal donors were assayed for their surface markers with mAb. The results are expressed as mean percentage ± SD, and the number of experiments is indicated in parentheses.

*The percentage of cells simultaneously reactive with OKT3 and Leu-11a mAb by two-color immunofluorescence staining

Table 3. Characterization of LAK Effector Cells by Negative Selection With mAb

Effector Cells	Duration of Culture	Target Cells	Treatment	Percent Yield of Cells	Percentage of Cytotoxicity E:T 80:1	Percentage of Cytotoxicity E:T 20:1
Patient 38	5 d	autologous	control	73	35	21
			OKT3	11	19*	14*
			Leu-11b	61	26*	21
Patient 40	5 d	autologous	control	73	21	20
			OKT3	16	8*	6*
			Leu-11b	57	8*	10*
Normal donor I	5 d	patient 1	control	91	27	18
			OKT3	12	7*	3*
			Leu-11b	69	19*	9*
Normal donor II	5 d	patient 36	control	91	50	42
			OKT3	12	37*	22*
			Leu-11b	69	44*	32*
Patient 28	2 wk	autologous	control	90	34	23
			OKT3	15	27*	17*
			Leu-11b	65	24*	18*
Normal donor I	2 wk	patient 36	control	90	25	15
			OKT3	11	14*	3*
			Leu-11b	72	18*	12*
Normal donor II	2 wk	patient 36	control	87	48	29
			OKT3	25	36*	21*
			Leu-11b	62	27*	12*
Patient 40	4 wk	autologous	control	51	26	24
			OKT3	16	14*	12*
			Leu-11b	40	20*	20*
Normal donor I	4 wk	patient 37	control	97	52	37
			OKT3	11	29*	17*
			Leu-11b	80	29*	18*
Normal donor II	4 wk	patient 37	control	90	67	50
			OKT3	40	59*	43*
			Leu-11b	63	59*	39*

PBMC from the patients and the two normal donors were cultured for five days, 2 weeks, and 4 weeks with IL-2-containing medium, and assayed for their cytotoxicity against autologous or allogeneic tumor cells after negative selection of $OKT3^+$ or $Leu\text{-}11b^+$ cells by the panning method.

*Percentage of cytotoxicity significantly different ($P < 0.05$) from that of control

medium in spite of retaining strong cytotoxicity for K562 target cells.[41] Therefore we focused on LAK, which has been shown to lyse autologous fresh solid tumor cells in more than 90% of the cancer patients tested,[3,4,12] and tested whether short-term (five-day) cultured LAK and IL-2-expanded (2- to 4-week cultured) LAK would also lyse hematologically malignant cells isolated from patients. The results show that short-term cultured and in vitro-expanded LAK lysed almost all types of leukemia and lymphoma cells tested in autologous and allogeneic effector-tumor systems. These

Table 4. Effect of mAb Treatment on Alloreactive Killer T Cells, LAK Cells, and Their Target Cells

Experiment	mAb Treatment	Final Concentration of mAb	Percentage of Cytotoxicity for Target Cells: PHA Blasts	Tumor Cells of Patient 21	Tumor Cells of Patient 36	Autologous PBMC
1	Control	—	34	60	68	13
	OKT3	1 µg/mL	10*	59	66	26*
	OKT4	1 µg/mL	29	56	67	13
	OKT8	1 µg/mL	24*	55	71	16
2	Control	—	32	46	61	12
	W6/32	1.50	21*	42	57	10
	OKIa1	1·50	33	57*	65*	18*

In experiment 1, effector cells were preincubated for 30 minutes with mAb prior to the addition of ^{51}Cr-labeled target cells, and, in experiment 2, ^{51}Cr-labeled target cells were preincubated for 30 minutes with mAb prior to the addition of effector cells.

Effector cells reactive for PHA blasts were alloreactive killer T cells induced after seven days of MLC. PHA blasts used as targets were obtained after three days' culture of stimulator's lymphocytes with 1:1,000-diluted PHA-P. Effector cells reactive for patients' tumor cells and autologous PBMC were LAK cells induced by five days' culture of normal donor's PBMC. Tumor cells of patients 21 and 36 expressed HLA class I and II antigens recognized by mAb W6/32 and OKIa1, respectively. E:T ratio in the cytotoxicity assay was 80:1 in all the experiments.

*Percentage of cytotoxicity significantly different ($P < 0.05$) from that of control.

Table 5. Chromosome Analysis of Fresh Bone Marrow Cells and IL-2–expanded PBMC

Donor	Fresh Bone Marrow Cells*	In Vitro–Cultured PBMC†	
		Duration of Culture	Chromosomes
Normal donor I	ND	2 wk	46, XY‡
		4 wk	46, XY
Normal donor II	ND	2 wk	46, XX‡
		4 wk	46, XX‡
Patient 2	46, XY	2 wk	46, XY
4	47, XX, +21	2 wk	46, XX‡
6	46, XX, t(8,21)	4 wk	46, XX (4 cells): 45, X, −X, t(6,8), 15q− (6 cells)
9	46, XY, t(15;17)	2 wk	46, XY
		4 wk	46, XY
12	47, X, −X, t(10q−,11q+,21q+), +3,+21	2 wk	46, XX
17	48, XX, +8, 20q−, +20q−	2 wk	46, XX
18	46, XY	2 wk	46, XY‡
19	46, XY, 10p−	2 wk	46, XY
20	46, XX, 6q−	2 wk	46, XX‡
		4 wk	46, XX

*Chromosome analysis of fresh bone marrow cells was done when the patients had active disease.

†PBMC from the patients were expanded with IL-2–containing medium when they were in complete remission, except for patient 12, who had active disease.

‡One or two cells showed nonclonal abnormalities

results suggest the potential application of LAK to clinical trials. However, the present study also demonstrated that this highly reactive LAK lysed autologous and allogeneic nonmalignant target cells as well to a low, but significant, degree, and that in vitro–expanded PBMC developed chromosomal changes of a clonal nature in a single patient.

Occasional induction of LAK reactive with nonmalignant target cells has been described by others.[3,42] Rosenberg et al[42] briefly mentioned that five- to seven-day cultured LAK expressed a low level of cytotoxicity for autologous blood lymphocytes and two- to three-day cultured LAK had only antitumor cytotoxicity. Therefore it is necessary in our experiments to elucidate the kinetics of development of LAK reactive with normal targets and to determine which subsets of normal cells are lysed by LAK.

As far as we know, there have been no previous reports concerning chromosomal analysis of LAK. Chromosomes of alloreactive human T cells, which had been maintained in IL-2–containing medium, were analyzed, and preliminary observations from several laboratories suggested that major chromosomal abnormalities are frequent in these cells.[43] Our patient 6 who had ANLL developed different abnormalities in her PBMC chromosomes after in vitro expansion (Table 5). Because chromosomes were not serially analyzed at the beginning of and throughout the culture period, it is not known when such change had occurred. Because she had been given combination chemotherapy before cultivation of PBMC, the possibility that the chemotherapy caused the chromosomal change cannot be ruled out. More detailed prospective studies are required as to when and how frequently chromosomal changes occur. The finding that the clonotypic abnormalities developed in a single patient should not be generalized to other patients.

It is worth noting that, throughout the culture period, PBMC were expanded without adding mitogens or antigens. Although a few reports indicate that the addition of Il-2 causes the proliferation of PBMC in short-term culture,[31,44,45] there have been no previous reports, to our knowledge, concerning in vitro expansion of PBMC in large numbers in longer term cultures, and their functional and phenotypic characterization. It is known that IL-2 mediates its proliferative effect on lymphocytes through its binding to IL-2 receptors,[46] IL-2 receptors being expressed after activation of lymphocytes by mitogens or antigens,[2,46] and that IL-2 receptors are present in less than 5% of resting lymphocytes.[47,48] Therefore it would be interesting to know whether IL-2 added in vitro stimulates the proliferation of PBMC already having IL-2 receptors, or whether IL-2 receptors are newly expressed and IL-2 receptor-bearing cells proliferate thereafter. The latter possibility was recently proved by Reem et al,[49] who demonstrated that IL-2 itself can induce and upregulate high-affinity IL-2 receptors on resting T lymphocytes. Overgrowth of T8-positive cells during the culture (Table 2) might be explained by the recent finding that T4-positive cells become refractory to IL-2 growth promotion earlier than T8-positive cells.[50]

Cells propagated in vitro with IL-2–containing medium were T3 and Leu-11 positive (Table 2). Since it was shown by two-color immunofluorescence staining that T3 antigen and Leu-11 antigen are not simultaneously expressed on the same cell, the proliferated cells consisted of two different populations of cells, namely, T cells and NK cells. Although surface markers of five-day cultured PBMC were not studied, it is highly likely that they contained two different populations as well, because in fresh uncultured PBMC and in 2-week cultured PBMC, T3-positive cells belong to different populations of cells from Leu-11–positive cells (Table 2). In 2-week and 4-week cultured PBMC, T8-positive cells proliferated. Our preliminary observation suggested that most of the T8-positive cells are included within the population of T3-positive cells by two-color immunofluorescence staining. Therefore it must be clarified whether these T8-positive cells have suppressor T-cell function.

Characterization of LAK effector cells revealed that both T and NK cells mediated LAK activity in autologous and allogeneic systems (Table 3) Because the number of T3-positive cells greatly exceeded that of Leu-11–positive cells in cultured PBMC (Tables 2 and 3), the level of cytotoxicity per single cell might be equal or higher in Leu-11–positive cells. With regard to the nature of LAK effector cells, conflicting results were reported by others.[4,29–33] Rosenberg's group reported that they are T cells,[4,29,30] Itoh et al[31] reported that they are NK cells, and Nishimura and Hashimoto,[32] and Merluzzi[33] reported that they are both T cells and NK cells These differences may be explained by the difference in target tumor cells used, concentration of IL-2, or length of the activation period. In contrast to freshly isolated effector cells, in which Leu-11–positive NK cells but not T3-positive T cells mediated cytotoxicity for K562 target cells,[40] both Leu-11–positive NK and T3-positive T cells mediated cytotoxicity for K562 and Raji cells, when PBMC cultured for five days, 2 weeks, and 4 weeks were used as effector cells (data not shown), and this finding could explain why the level of cytotoxicity for autologous tumor cells was roughly parallel to that of cytotoxicity for K562 and Raji cells (Fig 6).

The nature of receptors of LAK that recognize antigenic structures on target cells is not known, nor is that of antigenic structures on target cells CTL are known to recognize target cells via the Ti-T3 complex structure,[51] and CTL cytotoxicity is blocked by preincubation of CTL with mAb OKT3.[52] Cytotoxicity of T8-positive CTL that recognize HLA class I antigen is blocked by mAb OKT8 and mAb W6/32 which reacts with HLA class I antigen, whereas cytotoxicity of T4-positive CTL which recognize HLA class II antigen is blocked by mAb OKT4 and an Ab which reacts with HLA class II antigen.[53,54] As expected, cytotoxicity of alloreactive CTL was inhibited by mAb OKT3, OKT8, and W6/32 (Table 4). In contrast, LAK activity for patients' tumor cells or autologous PBMC was not inhibited by mAb treatment. These results suggest that LAK effector cells mediate their lytic function via different recognition mechanisms from alloreactive CTL. Thus T3-positive T cells mediating LAK activity (Table 3) seem to be true LAK effectors rather than IL-2–activated classical CTL. Interestingly, preincubation of LAK effector cells with OKT3 or of target cells with OKIa1 rather enhanced LAK activity against patients' tumor cells and autologous PBMC (Table 4). Although the results were reproducible, the reason for this enhancement was not clarified. With regard to the target structures recognized by LAK, Grimm and Wilson[55] have recently suggested that they are self-altered. As was suggested by the finding that nonmalignant target cells were less susceptible than malignant cells (Table 1), target structures appear to be present in larger quantity on malignant cells than on normal cells. It is worth noting that ALL cells in the most primitive stage of B-cell differentiation were more susceptible to allogeneic normal donors' LAK than those in more mature stages (Fig 3), and that lymphoma cells having clinical characteristics of high-grade malignancy were also more susceptible than those having characteristics of intermediate- and low-grade malignancy (Fig 4). Although such a tendency was not observed in the autologous effector-target system (Fig 5), this may be explained by the status of the patients at the time blood was taken. That is, LAK precursor cells derived from the patients may have been affected by various kinds of therapy or stage of the diseases.

LAK therapy seems to be very promising according to a recent report,[56] yet our present study raises some cautions as to its use. Can LAK cells be safely administered without lysing autologous normal cells? Do T8-positive cells propagated in vitro have suppressor T-cell functions? Do LAK cells contain cells with chromosomal abnormalities, and if so, how often, and what kind? We think that these problems need to be evaluated when LAK cells are used in clinical trials.

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REFERENCES

1 Morgan DA, Ruscetti FW, Gallo R· Selective in vitro growth of T lymphocytes from normal human bone marrows Science 193·1007, 1976

2. Ruscetti FW, Gallo RC Human T-lymphocyte growth factor: Regulation of growth and function of T lymphocytes. Blood 57:379, 1981

3. Lotze MT, Grimm EA, Mazumder A, Strausser JL, Rosenberg SA Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T-cell growth factor Cancer Res 41:4420, 1981

4 Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA Lymphokine-activated killer cell phenomenon Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2–activated autologous human peripheral blood lymphocytes J Exp Med 155·1823, 1982

5 Grimm EA, Ramsey KM, Mazumder A, Wilson DJ, Djeu JY, Rosenberg SA· Lymphokine-activated killer cell phenomenon II. Precursor phenotype is serologically distinct from peripheral T lymphocytes, memory cytotoxic thymus-derived lymphocytes, and natural killer cells. J Exp Med 157:884, 1983

6 Grimm EA, Robb RJ, Roth JA, Neckers LM, Lachman LB, Wilson DJ, Rosenberg SA Lymphokine-activated killer cell phenomenon III Evidence that IL-2 is sufficient for direct activation of peripheral blood lymphocytes into lymphokine-activated killer cells J Exp Med 158.1356, 1983

7 Rosenberg SA, Grimm EA, McGrogan M, Doyle M, Kawasaki E, Koths K, Mark DF· Biological activity of recombinant human interleukin-2 produced in *Escherichia coli.* Science 223 1412, 1984

8 Mazumder A, Rosenberg SA: Successful immunotherapy of

natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. J Exp Med 159:495, 1984

9. Mulé JJ, Shu S, Schwarz SL, Rosenberg SA: Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant interleukin-2 Science 225 1487, 1984

10 Mulé JJ, Shu S, Rosenberg SA The anti-tumor efficacy of lympholine-activated killer cells and recombinant interleukin 2 in vivo J Immunol 135:646, 1985

11 Lafreniere R, Rosenberg SA. Successful immunotherapy of murine experimental hepatic metastases with lymphokine-activated killer cells and recombinant interleukin 2 Cancer Res 45:3735, 1985

12 Rayner AA, Grimm EA, Lotze MT, Chu EW, Rosenberg SA: Lymphokine-activated killer (LAK) cells. Analysis of factors relevant to the immunotherapy of human cancer Cancer 55 1327, 1985

13 Bennett JM, Catovsky D, Daniel M, Flandrin G, Galton DAG, Granlnick HR, Sultan C Proposals for the classification of the acute leukemias. French-American-British (FAB) co-operative group Br J Haematol 33:451, 1976

14. National Cancer Institute sponsored study of classification of non-Hodgkin's lymphoma. Summary and description of a working formulation for clinical usage. Cancer 49 2112, 1982

15. Oshimi K, Aoyama M, Ohtani T, Okawa H, Yata J, Watanabe S, Mizoguchi H: A case report of T-cell lymphoma with suppressor phenotype and helper function for immunoglobulin synthesis Cancer 54.2029, 1984

16. Oshimi K, Akahoshi M, Hagiwara N, Tanaka M, Mizoguchi H: A case of T-cell chronic lymphocytic leukemia with an unusual phenotype and central nervous system involvement Cancer 55:1937, 1985

17. Nadler LM, Korsmeyer SJ, Anderson KC, Boyd AW, Slaughenhoupt B, Park E, Jensen J, Coral F, Mayer RJ, Sallan SE, Ritz J, Schlossman SF. B cell origin of non-T cell acute lymphoblastic leukemia. A model for discrete stages of neoplastic and normal pre-B cell differentiation. J Clin Invest 74.332, 1984

18. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF: Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 131:1789, 1983

19. Devos R, Plaetinck G, Cheroutre H, Simons G, Degrave W, Tavernier J, Remaut E, Fiers W: Molecular cloning of human interleukin 2 cDNA and its expression in E. coli Nucleic Acids Res 11.4307, 1983

20. Gillis S, Ferm MM, Ou W, Smith KA. T cell growth factor: Parameters of production and a quantitative microassay for activity. J Immunol 120:2027, 1978

21 Oshimi K, Oshimi Y, Satake M, Mizoguchi H: Natural killer-mediated lysis of normal and malignant target cells, and its regulation by monocytes. J Exp Med 162:472, 1985

22 Seeley JK, Golub SH. Studies on cytotoxicity generated in human mixed lymphocyte cultures. I. Time course and target spectrum of several distinct concomitant cytotoxic activities. J Immunol 120.1415, 1978

23 Oshimi K, Kano S, Sumiya M, Gonda N, Takaku F: Cytotoxicity of cultured mouse spleen cells against natural killer-sensitive target cells Characterization of the effector cells. Int Arch Allergy Appl Immunol 64:395, 1981

24. Forman J, Moller G. Generation of cytotoxic lymphocytes in mixed lymphocyte reactions I Specificity of the effector cells J Exp Med 138:672, 1973

25. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, Ziegle A: Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis Cell 14:9, 1978

26. Reinherz EL, Kung PC, Pesando JM, Ritz J, Goldstein G, Schlossman SF: Ia determinants on human T-cell subsets defined by monoclonal antibody Activation stimuli required for expression J Exp Med 150:1472, 1979

27. Okada M, Mizoguchi H, Kubota K, Nomura Y: Quantitative analysis of chromosomal G-bands in human hematopoietic disorders by methotrexate synchronization technique Cancer Genet Cytogenet 13:225, 1984

28. First International Workshop on Chromosomes in Leukemia. Chromosomes in acute nonlymphocytic leukemia Br J Haematol 39.311, 1978

29 Rosenstein M, Yron I, Kaufmann Y, Rosenberg SA: Lymphokine-activated killer cells: lysis of fresh syngeneic natural killer-resistant murine tumor cells by lymphocytes cultured in interleukin 2. Cancer Res 44:1946, 1984

30. Rayner AA, Grimm EA, Lotze MT, Wilson DJ, Rosenberg SA. Lymphokine-activated killer (LAK) cell phenomenon IV Lysis by LAK cell clones of fresh human tumor cells from autologous and multiple allogeneic tumors. J Natl Cancer Inst 75 67, 1985

31. Itoh K, Tilden AB, Kumagai K, Balch CM: Leu-11$^+$ lymphocytes with natural killer (NK) activity are precursors of recombinant interleukin 2 (rIL 2)–induced activated killer (AK) cells J Immunol 134 802, 1985

32. Nishimura T, Hashimoto Y: Induction of nonspecific killer T cells from non-immune mouse spleen cells by culture with interleukin 2. Gann 75:177, 1984

33. Merluzzi VJ Comparison of murine lymphokine-activated killer cells, natural killer cells, and cytotoxic T lymphocytes. Cell Immunol 95.95, 1985

34. Zarling JM, Bach FH. Continuous culture of T cells cytotoxic for autologous human leukemia cells Nature 280:685, 1979

35 Mukherji B, MacAlister TJ: Clonal analysis of cytotoxic T cell response against human melanoma. J Exp Med 158:240, 1983

36. DeVries JE, Spits H: Cloned human cytotoxic T lymphocyte (CTL) lines reactive with autologous melanoma cells I. In vitro generation, isolation and analysis to phenotype and specificity. J Immunol 132:510, 1984

37. Yssel H, Spits H, De Vries JE: A cloned human T cell line cytotoxic for autologous and allogeneic B lymphoma cells. J Exp Med 160.239, 1984

38. Uchida A, Micksche M: Lysis of fresh human tumor cells by autologous large granular lymphocytes from peripheral blood and pleural effusion Int J Cancer 32:37, 1983

39 Oshimi K, Oshimi Y, Motoji T, Kobayashi S, Mizoguchi H. Lysis of leukemia and lymphoma cells by autologous and allogeneic interferon-activated blood mononuclear cells. Blood 61.790, 1983

40. Oshimi K, Oshimi Y, Yamada O, Mizoguchi H. Lysis of lymphoma cells by autologous and allogeneic natural killer cells. Blood 65:638, 1985

41. Oshimi K, Oshimi Y, Naohara T, Mizoguchi H: Lysis of lymphoma cells by cultured large granular lymphocytes. Exp Hematol 13:1192, 1985

42 Rosenberg SA, Grimm EA, Lotze MT, Mazumder A: The growth of human lymphocytes in T cell growth factor: Potential applications to tumor immunotherapy, in Pick E (ed). Lymphokines (vol 7). Orlando, Fl, Academic Press, 1982, p 213

43. Johnson JP, Cianfriglia M, Glasebrook AL, Nabholz M. Karyotype evolution of cytolytic T cell lines, in Fathman G, Fitch FW (eds) Isolation and Utilization of T-Lymphocyte Clones Orlando, Fl, Academic Press, 1982, p 183

44. Trinchieri G, Matsumoto-Kobayashi M, Clark SC, Seehra J, London L, Perussia B Response of resting human peripheral blood natural killer cells to interleukin 2 J Exp Med 160:1147, 1984

45 Itoh K, Shiiba K, Shimizu Y, Suzuki R, Kumagai K: Generation of activated killer (AK) cells by recombinant interleukin 2 (rIL 2) in collaboration with interferon-γ (IFN-γ). J Immunol 134:3124, 1985

46. Robb RJ, Munck A, Smith KA. T cell growth factor receptors. Quantitation, specificity, and biological relevance. J Exp Med 154:1455, 1981

47. Uchiyama T, Broder S, Waldmann TA· A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. J Immunol 126:1393, 1981

48. Hofman FM, Modlin RL, Bhoopat L, Taylor CR Distribution of cells bearing the Tac antigen during ontogeny of human lymphoid tissue. J Immunol 134:3751, 1985

49. Reem GH, Yeh N-H, Urdal DL, Kilian PL, Farrar JJ: Induction and upregulation by interleukin 2 of high-affinity interleukin 2 receptors on thymocytes and T cells. Proc Natl Acad Sci USA 82:8663, 1985

50. Gullberg M, Smith KA: Regulation of T cell autocrine growth $T4^+$ cells become refractory to interleukin 2. J Exp Med 163:270, 1986

51 Meuer SC, Acuto O, Hussey RE, Hodgdon JC, Fitzgerald KA, Schlossman SF, Reinherz EL: Evidence for the T3-associated 90K heterodimer as the T-cell antigen receptor. Nature 303.808, 1983

52. Reinherz EL, Meuer S, Fitzgerald KA, Hussey RE, Levine H, Schlossman SF: Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. Cell 30:735, 1982

53. Meuer SC, Schlossman SF, Reinherz EL: Clonal analysis of human cytotoxic T lymphocytes: $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex regions. Proc Natl Acad Sci USA 79:4395, 1982

54. Landegren U, Ramstedt U, Axberg I, Ullberg M, Jondal M, Wigzell H: Selective inhibition of human T cell cytotoxicity at levels of target recognition or initiation of lysis by monoclonal OKT3 and Leu-2a antibodies. J Exp Med 155:1579, 1982

55 Grimm EA, Wilson DJ· The human lymphokine-activated killer cell system. V Purified recombinant interleukin 2 activates cytotoxic lymphocytes which lyse both natural killer-resistant autologous and allogeneic tumors and trinitrophenyl-modified autologous peripheral blood lymphocytes. Cell Immunol 94:568, 1985

56. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT, Seipp CA, Simpson C, Reichert CM Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med 313:1485, 1985

t(14;14)(q11;q32) in Biphenotypic Blastic Phase of Chronic Myeloid Leukemia

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A blastic crisis of chronic myeloid leukemia without a detectable chronic phase is reported. At diagnosis, blast cells present t(9;22)(q34;q11),t(14;14)(q11;q32) translocations and early B cell phenotype (DR +, TdT +, B4 +, BA1 +, J5 +). At relapse, the malignant clone evolves to a biphenotypic expression, the initial markers remain unchanged, and two myeloid antigens (My 7, My 9) appear. The wide overlap in percentages of blast cells displaying lymphoid and myeloid markers shows that a single clone bears antigens of both lineages. Simultaneous occurrence of a t(14;14)(q11;q32) translocation, usually found in T cell malignancies, and of a B cell phenotype raises the question of the relationship between chromosomal changes and surface marker expression. The malignant cell is assumed to be a progenitor cell, already committed to lymphoid lineage and retaining the potential to switch to myeloid lineage.

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THE (Ph^1) chromosome, which is the result of t(9;22)(q34;q11), is characteristic of chronic myeloid leukemia (CML), but it has also been described in 10% of adult acute lymphoblastic leukemias and in less than 5% of acute myeloid leukemias.[1] Karyotypes observed at remission allow discrimination between Ph^1-positive acute lymphoblastic leukemias (ALL) and the blastic phase of CML (BP-CML) without a detectable chronic phase. In remission of ALL, the majority of evaluable marrow cells do not show the Ph^1 chromosome, whereas in CML, most of the cells are Ph^1 positive.[2]

Blastic phases of CML are mostly of myeloid lineage and in one third of the cases of lymphoid lineage Further investigations have provided evidence of greater heterogeneity. Of all myeloid crises, some involve megakaryoblast and others erythropoietic cell lines.[3,4] In most cases, lymphoid crises show the same marker profile as common ALL,[4] and when differentiation antigens of B cells,[5] cytoplasmic immunoglobulin,[6,7] or immunoglobulin gene rearrangement[8] are found, they are clearly assigned to the B cell lineage. The presence of T cell markers in lymphoid blast crises has been documented.[9,10] Some authors[3,5,11-13] have mentioned mixed crises with two distinct populations of blast cells, but a biphenotypic blastic phase of CML with a single population of blast cells bearing both myeloid and lymphoid markers has seldom been reported.[9]

The t(14;14)(q11;q32) translocation is a common aberration in ataxia telangiectasia (AT), but it has also been described in lymphoproliferative disorders. These tandem translocations are especially associated with T cell malignancies.[14] Frequent involvement of the 14q11 breakpoint in T cell tumors has been recently correlated with the presence of the α-chain gene of the T cell receptor in this area of the chromosome 14.[15]

Here we report a blastic phase of CML, the cells of which presented t(9;22)(q34;q11) and t(14;14)(q11;q32) translocations, B cell phenotype at diagnosis, and biphenotypic (lymphoid-myeloid) expression at relapse.

CASE REPORT

A 68-year-old female was hospitalized for varicose vein surgery in January 1985. Presurgical analysis revealed an acute lymphoblastic leukemia. No splenomegaly, adenopathy, infection, or bleeding syndrome was present. White blood cell count at presentation was 130 × 10^9/L with 12% polymorphonuclear neutrophils, 57% lymphocytes, 5% monocytes, 26% blast cells, and 3% nucleated red blood cells Bone marrow analysis was consistent with ALL 90% of blast cells of lymphoid morphology (L_1 according to French-American-British classification); myeloperoxidase, Sudan black, and acid phosphatase were negative Informed consent for this and other standard diagnostic procedures, and approval of the CHU Toulouse/Purpan Human Protection Committee, were obtained The patient failed to respond to vincristine, cyclophosphamide, or prednisone therapy. With added daunorubicin, a short remission was obtained, and in March 1985, bone marrow sample analysis showed normal cellularity, 1% myeloblasts, 2% promyelocytes, 12% myelocytes, 14% metamyelocytes, 12% polymorphonuclear neutrophils, 1% eosinophils, 14% lymphocytes, 2% monocytes, 2% plasma cells, 35% erythroblasts, and 5% blast cells. At this time, the white blood cell count was 2.9 × 10^9/L with 90% neutrophils, 6% lymphocytes, 1% monocytes, 1% metamyelocytes, and 2% myelocytes.

Despite chemotherapy, blast cell numbers rose regularly, reaching 76% in bone marrow in June 1985. In September 1985, the white blood cell count was 460 × 10^9/L with 5% polymorphonuclear neutrophils, 2% lymphocytes, 2% myelocytes, 80% blast cells, and 11% promonocytes. The majority of blast cells retained the same lymphoid morphology, and myeloperoxidase reaction remained negative. This reaction was checked, using the ultrastructural method Sodium fluoride-sensitive esterase reaction was negative.

MATERIALS AND METHODS

Cytogenetic studies. Peripheral blood was cultured with phytohemagglutinin (PHA) for 96 hours Peripheral blood without stimulation and bone marrow were analyzed after 24 hours or after 72 hours (see Table 2). G banding was performed with the Seabright technique. Before harvesting, a sample of the culture medium was diluted (1.10 in RPMI), centrifuged on slides by cytospin, and stained by May-Grünwald-Giemsa. This study helped us to identify the type of dividing cell.

Immunologic studies. The leukemic cells used were either fresh or thawed at the time of the study. Viability of the sample on thawing was 90% as judged from the dye exclusion test, stained with

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Table 1. Reactivity of Monoclonal Antibodies

Monoclonal Antibody	Reactivity
B1 (Coultronics)	CD 20 (pan B)
B4 (Coultronics)	CD 19 (all B cells following Ia + pre-B cell)
BA$_1$ (Hybritech)	CD 24 (pan B, granulocytes)
BA$_2$ (Hybritech)	CD 9 (non-T, non-B cells; monocytes)
DR (Coultronics)	HLA class II DR
J5 (Coultronics)	CD 10 (CALLA)
MY 9 (Coultronics)	Myelomonocytic cells
MY 7 (Coultronics)	CDW13 (granulocytes, monocytes)
T101 (Hybritech)	CD 5 (pan T)
OKT11 (Ortho Diagnostics)	CD 2 (cells forming E rosettes)

According to the second workshop on leukocyte typing (Boston 1984)

trypan blue. The three samples were also centrifuged by cytospin and showed a 95% blast population An indirect immunofluorescent staining of cells in suspension was used. Briefly, 1 × 10^6 cells were treated with either 200 μL of a 1 160 dilution (for all antibodies, in a saturating dilution) of the specific monoclonal antibody to be tested, or 200 μL of a 1 160 dilution of an unreactive control antibody of an identical isotype After incubation and repeated washing, they were stained with a fluorescein-conjugated goat antimouse Ig antiserum (GAM-FITC Coultronics). After repeated incubation at 4 °C for 30 minutes, the cells were washed three times and analyzed. Analysis was performed on an EPICS C flow cytometer (Coulter Electronics, Hialeah, Fla) equipped with an argon ion laser For each sample, 10,000 cells were analyzed with a log amplifier. Windows on the display defining immunofluorescence-positive cells were set so that less than 2% of the cells stained with the unreactive control antibody were counted as positive Monoclonal antibodies used are summarized in Table 1.

RESULTS

Cytogenetic analysis Studies of peripheral blood cultured with PHA stimulation revealed a normal 46,XX karyotype. Karyotypes performed from bone marrow and blood without stimulation are listed in Table 2. Normal metaphases were never present. At diagnosis, a single clone with t(9;22)(q34;q11) and t(14;14)(q11;q32) translocations was identified. In remission, the t(14;14) translocation disappeared. A clone with Ph^1 alone was present. At the onset of relapse (relapse$_1$), the two former clones were simultaneously observed. In the terminal phase (relapse$_2$), a clonal evolution occurred. The only clone present was 46,XX,t(9;22)(q34;q11),t(14;14)(q11;q32),−5,+der(5) (5pter → q32::1qter → q23). A simultaneous study of the cytology and the cell karyotypes from the same culture medium showed that immature granulocytes bore only Ph^1, whereas blast cells bore both Ph^1 and t(14;14).

Immunologic studies. The results of the immunologic studies are listed in Table 3. At diagnosis, blasts had an early B cell phenotype (TdT, DR, B4, BA$_1$, J5). At relapse, TdT, DR, B4, BA$_1$, and J5 were found in percentages similar to those found in the first sample, and two myeloid antigens (My 7, My 9) appeared. The percentages of cells expressing lymphoid and myeloid markers widely overlapped. These results suggest a biphenotypic expression.

DISCUSSION

Our case shows t(9;22)(q34;q11),t(14;14)(q11;q32) in a biphenotypic (B lymphoid-myeloid) blastic phase of CML. Ph^1 and 14q+ have seldom been reported together. One instance[16] was non–T-, non–B-AL and another[17] was a lymphoid blast crisis of CML, the phenotype of which is unknown. Although our case was originally diagnosed as ALL, we can assume that it was actually a blastic phase of CML because normal metaphase has never been observed. During the short remission period, Ph^1 did not disappear, whereas the tandem translocation did. At this time, all hematopoietic cell lines were represented and only 5% of blast cells were present. Immature granulocytes (but not blast cells) are the probable bearers of this Ph^1 (see Table 2). Such findings argue in favor of CML rather than ALL, since granulocytes are not thought to be involved in the Ph^1-positive ALL. At the time of diagnosis, the blast population bearing both t(9;22)(q34;q11) and t(14;14)(q11;q32) translocations was quite homogeneous. Ninety percent of the blast cells were present, all suggesting lymphoid lineage (morphology and cytochemistry). This hypothesis was confirmed by immunologic characterization: the association of DR, TdT, B4, BA$_1$, and J5 markers, all present in high percentages, made the lymphoid nature unequivocal and was strongly suggestive of B cell lineage at an early stage of differentiation. This result is not surprising and is consistent with former findings reported in literature, that most BP-CML express the same phenotype as common ALL, and thus are usually of B cell lineage. Marker profiles observed in such malignancies represent the early stages of normal B cell maturation at or before the time of light chain rearrangement. The association of DR, B4, and J5 is the stage 3 of such a maturation (stage 1, DR alone; stage 2, DR, B4, stage 4, DR, B4, J5, B1). At relapse, two myeloid antigens (My 7, My 9)[18,19] appeared beside the unchanged initial markers. These two markers are restricted to myelomonocytic lineage and usually allow differentiation between lymphoid and

Table 2. Karyotypes and Types of Dividing Cells

	Material Used	No of Cells Studied			Karyotype	Cell Cytology in Mitosis at Time of Karyotype
		Total	Normal	Abnormal		
Diagnosis, January 1985	Bone marrow after 24 hours	16	0	16	46,XX,t(9,22)(q34,q11),t(14,14)(q11,q32)	Only blast cells
Remission, March 1985	Bone marrow after 72 hours	28	0	28	46,XX,t(9,22)(q34,q11)	Only immature granulocytes
Relapse$_1$, June 1985	Bone marrow after 24 hours	32	0	26 6	46,XX,t(9,22)(q34,q11),t(14,14)(q11,q32) 46,XX,t(9,22)(q34,q11)	Both blast cells and immature granulocytes
Relapse$_2$, September 1985	Peripheral blood without stimulation after 24 hours	34	0	34	46,XX,t(9,22)(q34,q11),t(14,14)(q11,q32) −5,+der(5)(5pter → q32 1qter → q23) (see Fig 1)	Only blast cells

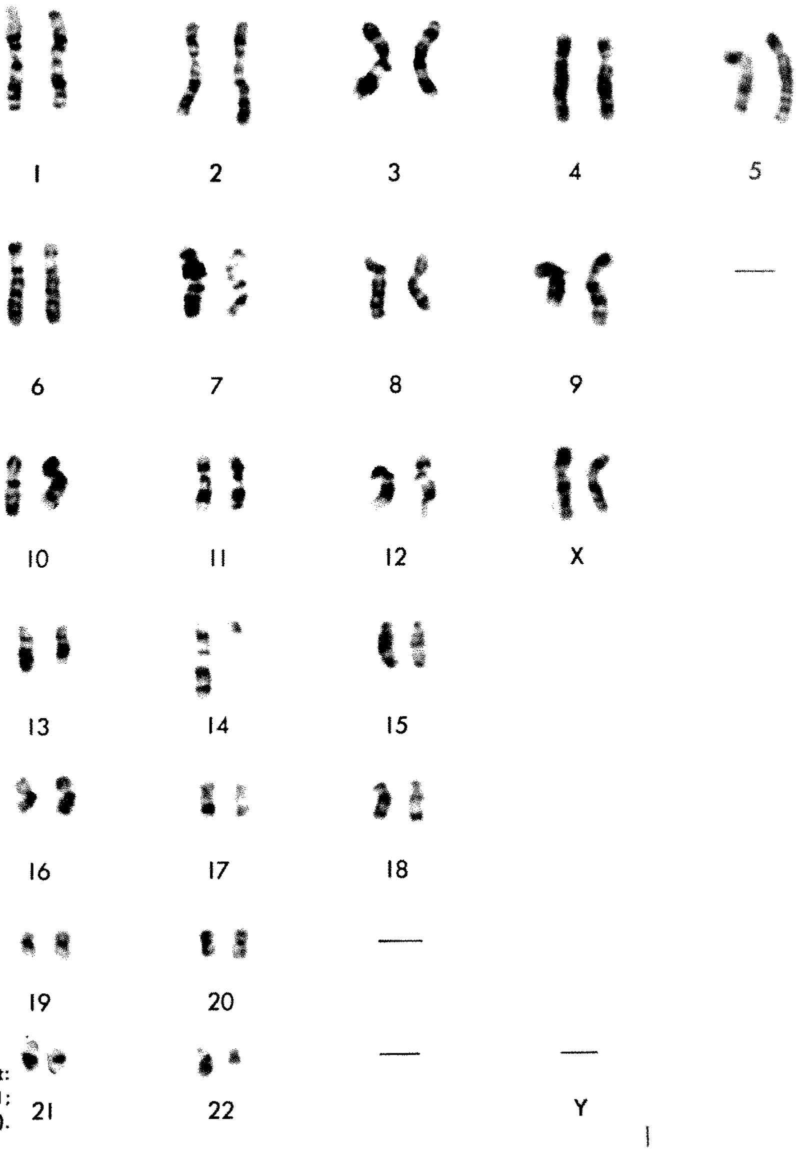

Fig 1. G-banded karyotype of the patient: 46, XX, t(9; 22)(q34; q11), t(14; 14)(q11; q32), −5, +der(5)(5pter → q32::lqter → q23).

myeloid leukemias. The wide overlap in percentages of positive cells with myeloid and lymphoid markers suggests that blast cells simultaneously exhibit antigens of both lineages. The morphology of blast cells did not show any sign of myeloid differentiation during the phenotypic evolution. Later, in the terminal phase of relapse, a minor population of promonocytes appeared. Myeloperoxidase reaction and sodium fluoride-sensitive esterase reaction remained negative throughout the evolution. At the onset of relapse, a major clone with both t(9;22)(q34;q11) and t(14;14)(q11;q32) reappeared, followed in the terminal phase by an additional change involving chromosomes 1 and 5 in this clone. Thus, the presence of identical cytogenetic markers at diagnosis and relapse shows that the same malignant clone evolved from a classic early B precursor phenotype to a biphenotypic lymphoid-myeloid phenotype.

The translocation t(14;14)(q11;q32) has been observed in AT and is also closely related to T cell disorders. It has been found in T cell lymphoma,[20] in the Sézary syndrome,[21] and in T cell CLL.[22,23] In the latter, as well as in the case reported by

Table 3. Expression of Differentiation Markers

Surface Marker	Positive Cells (%) Bone Marrow Diagnosis	Positive Cells (%) Bone Marrow Relapse$_1$	Positive Cells (%) Peripheral Blood Relapse$_2$
B1	1	1	4
B4	80	95	72
BA$_1$	80	66	34
BA$_2$	ND	47	61
DR	96	97	95
T101	ND	6	13
T11	ND	7	4
J5	90	91	58
MY 7	ND	94	70
MY 9	0	85	80
TdT	Positive	Positive	Positive
cIg	Negative	Negative	Negative

ND, not done; cIg, cytoplasmic immunoglobulin.

McCaw et al,[24] the malignant clone has emerged from a preexisting AT lymphocyte clone. This tandem translocation affects the 14q11 region of chromosome 14, in which the α-chain gene of the T cell receptor was mapped.[15] In fact, a molecular analysis of translocations with a breakpoint in 14q11 has seldom been carried out. In a recent report[25] concerning one case of T acute leukemia with a t(11;14)(p13;q11) translocation, the α-chain gene has been found to be split by the chromosomal breakpoint. Our case shows a simultaneous occurrence of B cell markers and a translocation usually found in T malignancies, thus raising the question of the relationship between chromosomal changes and surface marker expression. No history of AT was reported in the patient's family. Acid phosphatase reaction was negative and the percentages of the two T cell markers studied are too low to indicate a T cell phenotype. Unfortunately, we could not study a more immature marker, such as a CD_7.[26] Considering the different definitions of chromosomal techniques (>5,000 kb) and of molecular analyses (<50 kb), we can interpret our case as an exception in which, despite a typical aspect of tandem translocation, the breakpoint occurs far from the α-chain gene locus. Alternatively, we can hypothesize that even if the breakpoint does occur within the α-chain gene, it may be an ineffective rearrangement, without affecting the T cell activation.

The significance of the biphenotypic expression remains unclear. It has been interpreted as an aberrant gene expression of malignant cells.[27,28] Alternatively, the question arises of whether or not it may represent a clonal expansion of a normal counterpart. In our case, the third chromosomal change involving chromosomes 1 and 5 cannot account for the expression of myeloid antigens because this change occurred only in the terminal phase (relapse$_2$), 3 months after the phenotypic evolution. Because of the constant presence of Ph^1, a reliable marker of stem cells, we can assume that this case represents an early precursor recently committed to B cell lineage but retaining the potential to switch to myeloid lineage. The expression of My 7 and My 9 antigens, usually found on granulocytic and monocytic lineages as early as the CFU-C stage,[18,19] may reflect this potential switch. Furthermore, the emergence of promonocytes, only detectable by morphology, may also indicate a monocytic process of differentiation. Only a molecular approach, together with an immunophenotypic and a karyotypic study, will allow a better understanding of such puzzling cases and the relationship between gene rearrangement and gene function.

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REFERENCES

1. Hagemeijer A: Le chromosome Philadelphie, 25 ans après 1960–1985. Nouv Rev Fr Hematol 27:153, 1985
2. Catovsky D: Ph^1 positive acute leukaemia and chronic granulocytic leukaemia: One or two diseases? Br J Haematol 42:493, 1979
3. San Miguel JF, Tavares De Castro J, Matutes E, Rodriguez B, Polli N, Zola H, McMichael AJ, Bollum FJ, Thompson DS, Goldman JM, Catovsky D: Characterization of blast cells in chronic granulocytic leukaemia in transformation, acute myelofibrosis and undifferentiated leukaemia. II. Studies with monoclonal antibodies and terminal transferase. Br J Haematol 59:297, 1985
4. Bettelheim P, Lutz D, Majdic O, Paietta E, Haas O, Linkesch W, Neumann E, Lechner K, Knapp W: Cell lineage heterogeneity in blast crisis of chronic myeloid leukaemia. Br J Haematol 59:395, 1985
5. Griffin JD, Todd RF, Ritz J, Nadler LM, Canellos GP, Rosenthal D, Gallivan M, Beveridge RP, Weinstein H, Karp D, Schlossman SF: Differentiation patterns in the blastic phase of chronic myeloid leukaemia. Blood 61:85, 1983
6. Le Bien TW, Hozier J, Minowada J, Kersey JH: Origin of chronic myelocytic leukemia in a precursor of pre-B lymphocytes. N Engl J Med 301:144, 1979
7. Greaves MF, Verbi W, Reeves BR, Hoffbrand AV, Drysdale HC, Jones L, Sacker LS, Samaratunga I: Pre-B phenotypes in blast crisis of Ph^1 positive CML: Evidence for a pluripotential stem cell "target." Leuk Res 3:181, 1979
8. Bakhshi A, Minowada J, Arnold A, Cossman J, Jensen JP, Whang-Peng J, Waldmann TA, Korsmeyer SJ: Lymphoid blast crises of chronic myelogenous leukemia represent stages in the development of B-cell precursors. N Engl J Med 309:826, 1983
9. Griffin JD, Tantravahi R, Canellos GP, Wisch JS, Reinherz EL, Sherwood G, Beveridge RP, Daley JF, Lane H, Schlossman SF: T-cell surface antigens in a patient with blast crisis of chronic myeloid leukemia. Blood 61:640, 1983
10. Herrmann F, Komischke B, Kolecki P, Ludwig WD, Sieber G, Teichmann H, Rühl H: Ph^1 positive blast crisis of chronic myeloid leukaemia exhibiting features characteristic of early T blasts. Scand J Haematol 32:411, 1984
11. Janossy G, Woodruff RK, Paxton A, Greaves MF, Capellaro D, Kirk B, Innes EM, Eden OB, Lewis C, Catovsky D, Hoffbrand AV: Membrane marker and cell separation studies in Ph^1-positive leukemia. Blood 51:861, 1978
12. Janossy G, Woodruff RK, Pippard MJ, Prentice G, Hoffbrand AV, Paxton A, Lister TA, Bunch C, Greaves MF: Relation of "lymphoid" phenotype and response to chemotherapy incorporating vincristine-prednisolone in the acute phase of Ph^1-positive leukemia. Cancer 43:426, 1979
13. Parkin JL, McKenna RW, Brunning RD: Philadelphia chromosome-positive blastic leukaemia: Ultrastructural and ultracytochemical evidence of basophil and mast cell differentiation. Br J Haematol 52:663, 1982
14. Ueshima Y, Rowley JD, Variakojis D, Winter J, Gordon L: Cytogenetic studies on patients with chronic T cell leukemia/lymphoma. Blood 63:1028, 1984
15. Croce CM, Isobe M, Palumbo A, Puck J, Ming J, Tweardy D, Erikson J: Gene for α-chain of human T-cell receptor: Location on chromosome 14 region involved in T-cell neoplasms. Science 227:1044, 1985
16. Maekawa T, Sonoda Y, Taniwaki M, Misawa S, Abe T, Takino T: Ph^1-positive acute lymphoblastic leukemia with a 14q+ chromosome abnormality. Blood 63:310, 1984
17. Carbone P, Barbata G, Caronia F: Marker chromosome 14q+ in a case of Ph^1-positive chronic myeloid leukemia in lymphoid blastic crisis at presentation. Acta Haematol 67:230, 1982
18. Griffin JD, Ritz J, Beveridge RP, Lipton JM, Daley JF, Schlossman SF: Expression of MY7 antigen on myeloid precursor cells. Int J Cell Cloning 1:33, 1983
19. Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8:521, 1984
20. Gaeke ME, Vardiman JW, Miller W, Medenica M, Hopper

JE, Rowley JD: Human T-cell lymphoma with suppressor effects on the mixed lymphocyte reaction (MLR). I. Morphological and cytogenetic analysis. Blood 57:643, 1981

21. Shah-Reddy I, Mayeda K, Mirchandani I, Koppitch FC: Sézary syndrome with a 14:14(q12;q31) translocation. Cancer 49:75, 1982

22. Levitt R, Pierre RV, White WL, Siekert RG: Atypical lymphoid leukemia in ataxia telangiectasia. Blood 52:1003, 1978

23. Sparkes RS, Como R, Golde DW: Cytogenetic abnormalities in ataxia telangiectasia with T-cell chronic lymphocytic leukemia. Cancer Genet Cytogenet 1:329, 1980

24. McCaw BK, Hecht F, Harnden DG, Teplitz RL: Somatic rearrangement of chromosome 14 in human lymphocytes. Proc Natl Acad Sci USA 72:2071, 1975

25. Erikson J, Williams DL, Finan J, Nowel PC, Croce CM: Locus of the α-chain of the T-cell receptor is split by chromosome translocation in T-cell leukemias. Science 229:784, 1985

26. Haynes BF, Metzgar RS, Minna JD, Bunn PA: Phenotypic characterization of cutaneous T-cell lymphoma. Use of monoclonal antibodies to compare with other malignant T cells. N Engl J Med 304:1319, 1981

27. Bettelheim P, Paietta E, Majdic O, Gadner H, Schwarzmeier J, Knapp W: Expression of a myeloid marker on TdT-positive acute lymphocytic leukemic cells: Evidence by double-fluorescence staining. Blood 60:1393, 1982

28. Smith LJ, Curtis JE, Messner HA, Senn JS, Furthmayr H, McCulloch EA: Lineage infidelity in acute leukemia. Blood 61:1138, 1983

CONCISE REPORT

Recipient Immune-Competent T Lymphocytes Can Survive Intensive Conditioning for Bone Marrow Transplantation

By Anna Butturini, Robert C. Seeger, and Robert P. Gale

Bone marrow transplantation is usually preceded by intensive chemotherapy and radiation therapy designed to completely eliminate recipient immune-competent cells that might reject the donor bone marrow. We show that seven of 14 bone marrow transplant recipients who received intensive conditioning retained circulating T lymphocytes that proliferate after incubation with interleukin 2 and phytohemagglutinin and function as effector cells in an in vitro model of graft rejection. These T cells may mediate graft rejection.

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FAILURE of engraftment and graft rejection are important complications of bone marrow transplantation in humans. Graft failure occurs in 20% to 40% of individuals with aplastic anemia who receive HLA-identical transplants following pretransplantation conditioning with cyclophosphamide.[1] The incidence is even higher, 40% to 70%, in recipients of HLA-mismatched grafts.[2] Graft failure/rejection also occurs in recipients of bone marrow transplants for leukemia: graft rejection develops in approximately 1% of recipients of HLA-identical grafts and 15% of HLA-mismatched recipients.[3] These incidences increase substantially, to 15% and 30%, when T lymphocytes are removed from the transplanted bone marrow in attempts to prevent graft-*v*-host disease.[4]

The precise mechanism of graft failure/rejection is unknown, and there are no accurate in vitro tests to predict or define the mechanisms involved. Considerable indirect data suggest an immune basis of graft rejection in most instances. These include the observations that graft failure can be prevented or substantially reduced using more intensive pretransplant immune suppression[5,6] and that a lower incidence of graft rejection is reported in untransfused patients.[7] Extensive in vitro data and studies in animals including dogs and subhuman primates support the hypothesis that graft rejection is immune mediated.[8-11]

Despite these data that strongly suggest an immune basis of graft failure/rejection, there is no direct evidence in humans that immune-competent lymphocytes survive the pretransplant conditioning regimen. Most transplant patients receive high doses of drugs and radiation that are presumed to eradicate recipient lymphocytes completely and irreversibly. However, recent data in monkeys indicate that clonogenic T lymphocytes are present in peripheral blood following high-dose total-body irradiation, similar to that used in humans for pretransplant conditioning.[12]

We studied the proliferative and functional properties of peripheral blood lymphocytes in transplant patients after intensive conditioning regimens. In half of the patients it was possible to recover considerable numbers of T lymphocytes able to proliferate in vitro in the presence of phytohemagglutinin (PHA) and recombinant interleukin 2 (IL 2). By expanding these cells in long-term culture, we showed that they exibit many of the immunologic characteristics of normal T cells such as the ability to proliferate in mixed lymphocyte culture (MLC) and to develop into cytotoxic effector cells in an in vitro model of graft rejection.

MATERIALS AND METHODS

Fourteen consecutive patients were studied, six with acute lymphoblastic leukemia, five with acute myelogenous leukemia, two with chronic myelogenous leukemia, and one with advanced Hodgkin's lymphoma. Clinical details are indicated in Table 1.

Ten to 20 mL of peripheral blood was obtained within 24 hours of the last dose of radiotherapy and prior to the infusion of donor bone marrow cells. Informed consent was obtained from all individuals. Mononuclear cells were isolated on a Ficoll-Hypaque gradient, washed three times, and resuspended in RPMI 1640 supplemented with glutamine, antibiotics, 12% fetal calf serum, 20 mg/100 mL bovine albumin, 10 mg/100 mL human transferrin, and 0.1% of 0.1 mol/L $FeCl_3$. Viability of mononuclear cells, tested by trypan blue dye exclusion, was >95%.

Cultures were established using 5 to 10 × 10^4 cells per well in 24-well plates in 1.5 mL of medium in the presence of 1% PHA (Wellcome Reagents, Research Triangle Park, NC) and 100 U/mL of recombinant IL 2 (Biogen Research Corp, Cambridge, Mass). Culture medium was replaced on days 4, 9, and 15 with fresh medium supplemented with PHA and IL 2, and weekly thereafter with medium and IL 2. On days 4, 9, and 15, aliquots of cells were counted and analyzed for morphology and phenotyped using T3, T11, T4, and T8 monoclonal anti-T lymphocyte antibodies (Coulter Immunology, Hialeah, Fla) by immunoperoxidase staining. Between day 15 and 25 when cell numbers exceeded 2 × 10^6, half of the cells were washed twice, incubated for 24 hours in medium alone, and assayed in MLC[13] with irradiated allogenic stimulator cells. Cells generated in MLC were tested for cytotoxicity against PHA-stimulated, ^{51}Cr-labeled allogenic lymphocytes.[14] Natural killer (NK) activity was tested using K562 as target cells.[15] Clonal growth of lymphocytes was assayed in semisolid matrix, and T colonies (>40 cells) were quantitated using an inverted microscope after ten days of culture.[16]

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Table 1. Patient Characteristics and Lymphoid Growth

UPN	Age (yr)	Sex	Disease	Chemotherapy	TBI (Gy)	TLI	Lymphoid Growth
463	7	F	AML, 2 rem	CTX + Ara-C	10	no	yes
464	33	F	AML, 1 rem	Ara-C	13.50	yes	no
465	5	M	ALL, rel	CTX + Ara-C	10	no	no
466	18	M	ALL, 1 rem	Ara-C	13.50	yes	yes
467	25	F	AML, 2 rem	Ara-C	13.50	yes	yes
468	19	M	ALL, rel	VBL + CTX	11.25	no	no
469	16	M	ALL, 2 rem	Ara-C	13.50	yes	yes
470	31	F	CML, cp	Ara-C	13.50	yes	yes
471	20	M	ALL, 2 rem	Ara-C	13.50	yes	yes
472	22	M	AML, 2 rem	Ara-C	13.50	yes	no
473	30	M	ALL, rel	VBL + CTX	11.25	no	no
474	23	M	CML, acc	CTX	13.50	yes	no
475	34	F	Hodgkin's	VBL + CTX	11.25	no	no
476	39	M	AML, 1 rem	Ara-C	13.50	yes	yes

Abbreviations: UPN, unique patient number; rem, remission; rel, relapse; cp, chronic phase; acc, accelerated phase; TBI, total-body irradiation; TLI, total-lymphoid irradiation (2.25 Gy); CTX, Cytoxan (cyclophosphamide); Ara-C, cytosine arabinoside; VBL, vinblastine.

RESULTS

Lymphoid cell cultures were established from seven patients for periods of 29 to 52 days. Continuous stimulation with IL 2 was required for cell proliferation. Cells from the other patients showed little or no growth.

The initial proliferation was characterized by cells with abnormal morphology. These cells stained strongly with T11 and T3 but weakly with T8 and T4. After ten days, the cells were replaced by a population with the morphology of normal IL 2–activated lymphocytes. The lymphoid origin of these cells was further established by reactivity with T11, T3, T4, and T8. The T8/T4 ratios in most cultures were 3 to 1.5:1. T cells from five patients (UPN 466, 467, 470, 471, and 476) formed colonies in semisolid matrix; the plating efficiency was 10^{-1} to 10^{-2}.

T cells from four of five cultures proliferated when cocultured with allogeneic irradiated stimulator cells. Cells from three of four MLCs were cytotoxic for ^{51}Cr-labeled target cells syngeneic to the stimulator cells (specific release: 12%, 16%, and 29%). None of seven lymphocyte cultures tested was directly cytotoxic for ^{51}Cr-labeled K562 cells, indicating an absence of detectable NK activity.

DISCUSSION

This study indicates that viable and potentially functional T lymphocytes of both helper (T4) and suppressor (T8) phenotype survive intensive pretransplant conditioning regimens. These cells are able to proliferate after exposure to PHA and IL 2, react to disparate HLA antigens in MLC, and exhibit cell-mediated cytotoxicity. NK activity was absent. Considering the number of T lymphocytes in the peripheral blood at the time of the study (0.002 to 0.008×10^9/L) and the plating efficacy (10^{-1} to 10^{-2}), it is possible that a 70-kg recipient has at least 0.1 to 2.5×10^6 lymphocytes that can proliferate and react against the allogeneic donor bone marrow cells.

In most instances, the limited number of cells that survive the conditioning regimen are probably eliminated by donor immune-competent cells in the graft. When T cells are removed from the transplanted bone marrow, these residual host T cells may survive and react against the donor bone marrow. Our observations provide a possible explanation of graft failure/rejection occurring after bone marrow transplantation in humans. Furthermore, they suggest that graft rejection may be increased in T cell–depleted bone marrow transplantation or overcome by more intensive immune suppression. Preliminary data supporting both hypotheses are available.[4]

The approach we describe may aid in developing more effective immunosuppressive conditioning regimens since the number and reactivity of residual T cells can be determined. Ultimately, studies of this nature may clarify the cellular mechanism underlying graft rejection.

REFERENCES

1. Storb R, Witherspoon RP, Sullivan KM, Stewart P, Sanders JE, Sale GE, Prentice RL, Hansen JA, Doney K, Deeg HJ, Clift RA, Buckner CD, Appelbaum FR, Thomas ED: Allogeneic marrow transplant for treatment of severe aplastic anemia, in Gale RP (ed): Recent Advances in Bone Marrow Transplantation. New York, Liss, 1983, p 3

2. Bortin MM, Gale RP, Rimm AA: Allogeneic bone marrow transplantation for 144 patients with severe aplastic anemia. JAMA 245:1132, 1981

3. Gale RP, Champlin RE: Bone marrow transplantation in leukemia: Critical analysis and controlled clinical trials, in Gale RP (ed): Recent Advances in Bone Marrow Transplantation. New York, Liss, 1983, p 71

4. Gale RP: T cells, bone marrow transplantation and immunotherapy, in Fahey JL (moderator): Immune Intervention Disease. Ann Intern Med 1986 (in press)

5. Gale RP, Ho W, Feig S, Champlin R, Tesler A, Arenson E, Ladish S, Young L, Winston D, Sparkes R, Fitchen J, Territo M, Sarna G, Wong L, Paik Y, Brison Y, Golde D, Fahey J, Cline M: Prevention of graft rejection following bone marrow transplantation. Blood 57:9, 1981

6. Smith BR, Guinan EC, Parkman R: Efficacy of a cyclophos-

phamide-procarbazine-antithymocyte serum regimen for prevention of graft rejection following bone marrow transplantation for transfused patients with aplastic anemia. Transplantation 39:671, 1985

7. Storb R, Thomas ED, Buckner CD, Clift RA, Deeg HJ, Fefer A, Goodell BW, Sale GE, Sanders JE, Singer J, Stewart P, Weiden PL: Marrow transplantation in thirty "untransfused" patients with severe aplastic anemia. Ann Intern Med 92:30, 1980

8. Storb R, Weiden PL, Deeg HL, Graham TC, Atkinson K, Slicher SJ, Thomas ED: Rejection of marrow from DLA identical canine littermates given transfusion before grafting: Antigens involved are expressed on leukocytes and skin epithelial cells but not on platelets and red blood cells. Blood 54:477, 1979

9. Dallman MJ, Mason DW: Which T cells cause graft rejection? Transplantation 33:217, 1985

10. Steinmuller D: Which T cells cause allograft rejection? Transplantation 40:229, 1985

11. Wagenmaker G: Problems and prospects of histoincompatible bone marrow transplantation studied in Rhesus monkeys, in Hagenbeek A, Löwenberg B (eds): Minimal Residual Disease in Acute Leukemia. Dordrecht, Nijhoff, 1986, p 363 (abstr)

12. Reisner Y, BenBasat I, Douer D: A primate pre-clinical model for bone marrow transplantation: Definite proof for host radioresistant clonable T cells. Exp Hematol 13:321, 1985

13. Opelz G, Kuichi M, Takasuki M: Reactivity of lymphocyte subpopulation in human mixed lymphocyte culture. J Immunogenet 2:1, 1975

14. Schwartz A, Sutton SL, Gershan RK: Regulation of in vitro cytotoxic T lymphocyte generation 1: Evidence that killer cell precursors differentiate to effector cells in two steps. J Exp Med 155:783, 1982

15. Timonen T, Saksela E, Raucki A, Hayvey P: Fractionation, morphological and functional characterization of effector cells responsible for human natural killer activity against cell line targets. Cell Immunol 48:133, 1979

16. Goube de Laforest P, Thomas P, Pelletier D, Tanzer J: Studies on cell interactions using a micromethod for growing T-lymphocyte colonies in agar culture. Immunology 38:561, 1979

CONCISE REPORT

Rearrangement of the *bcr* Gene in Philadelphia Chromosome–Negative Chronic Myeloid Leukemia

By T.S. Ganesan, F. Rassool, A-P. Guo, K.H. Th'ng, C. Dowding, J.A. Hibbin, B.D. Young, H. White, T.O. Kumaran, D.A.G. Galton, and J.M. Goldman

We studied the clinical, hematologic, cytogenetic, and molecular biologic features of seven patients with Philadelphia (Ph^1) chromosome–negative chronic myeloid leukemia (CML). In five cases the hematologic findings were indistinguishable from those of patients with classical Ph^1-positive disease. Myeloid cells were studied by chromosome-banding techniques. One patient had a masked Ph^1 chromosome (with translocation t(4;9;22)), one had a deletion involving chromosome 16, and one had a small minority population of 22q− cells without 9q+ but otherwise normal metaphases; metaphases from the other four patients were entirely normal. DNA prepared from the myeloid cells was digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Bam*HI and *Bgl*II. Southern analysis using a 0.6-kb fragment of the breakpoint cluster region (*bcr*) gene showed the presence in each patient's DNA of a germline fragment together with a rearranged fragment or fragments with at least one of the restriction enzymes. We conclude that genomic changes in the *bcr* gene characteristic of CML can be present in the absence of a Ph^1 chromosome.

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THE MAJORITY of patients with chronic myeloid leukemia (CML) have a characteristic deletion of a portion of the long arm of one chromosome 22, the Philadelphia (Ph^1) chromosome, in their myeloid cells. The missing material is translocated to chromosome 9 such that the usual karyotype is described as t(9;22)(q34;q11). In Ph^1-positive CML the oncogene (c-*abl*) normally present on chromosome 9 is translocated to chromosome 22[1,2] where it comes into juxtaposition with the 5′ portion of a specific sequence of DNA named the breakpoint cluster region (*bcr*).[3] A chimeric c-*abl*-related mRNA has been identified in cells from patients with CML[4,5] and is associated with the presence of a fusion protein that has tyrosine kinase activity.[6] Thus patients with Ph^1-positive CML show evidence of rearrangement of DNA within the *bcr* region.

About 10% of patients regarded on clinical and hematologic grounds as having CML lack the Ph^1 chromosome. Some of these have a leukemia that is totally indistinguishable from Ph^1-positive disease in all other respects, whereas in other cases subtle clinical or hematologic features suggest that the disease is different. We report results of studying the clinical, hematologic, cytogenetic, and molecular biologic features in seven patients with Ph^1-negative CML.

MATERIALS AND METHODS

Patients. We selected for study seven patients who had attended the Hammersmith Hospital within the last 2 years with a diagnosis of Ph^1-negative CML. Their clinical and hematologic features at diagnosis are shown in Table 1.

Granulocyte-macrophage progenitor cell (CFU-GM) assay. Mononuclear cells from the peripheral blood of five of the seven patients were cultured in a standard methylcellulose system for CFU-GM with mixed lymphocyte culture–conditioned medium as the source of colony-stimulating activity.[7] Colonies consisting of 50 or more cells were scored after incubation for 12 days.

Cytogenetic analysis. Cytogenetic studies were carried out on fresh bone marrow cells or on fresh or cryopreserved buffy coat cells collected from the patients at the time of diagnosis. Chromosomes were prepared according to standard methods and analyzed after Giemsa banding.[8]

bcr *gene rearrangement.* Leukemic cells were isolated from the peripheral blood or marrow samples, and high-molecular weight

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Table 1. Clinical and Hematologic Details of Seven Patients With Ph^1-Negative CML

			Peripheral Blood at Diagnosis													CFU-GM per 10^6 Cells (±SEM)	Treatment and Response
						Differential (Percentage of 300 Cells)											
Patient	Sex/ Age	Spleen* (cm)	Hb (g/dL)	WBC (× 10^9/L)	Platelets (× 10^9/L)	Bl	Pm	My + Met	Neut	Eos	Baso	Mono	Lymph	NRBCs	NAP		
1	M/24	25+	8.3	174	941	6	6	21	41	6	14	0	4	0	0	360 ± 48	HU, PR
2	M/58	0	11.2	170	53	1	3	28	61	0	0.5	0.5	6	0	0-12	333 ± 30	HU, PR
3	M/31	0	12.1	46	375	0	0	10	56	17	2	1	14	0	NA	340 ± 80	BU, HU, CR, BMT
4†	F/33	0	10.5	77	492	0	0.5	14	67	2	1	7	8.5	0	0	ND	BU, HU, CR, BMT
5†	M/62	3	10.6	105	87	0	4	31	58	0	0	1	2	2	NA	ND	SRT, CR
6	F/43	12	10.3	506	319	2	4	28	59	2	3	1	1	0.3	0	324 ± 105	BU, CR
7	F/54	3	7.0	163	545	2	4	22	58	2	9	1	1	0.3	0	4,100 ± 425	BU, HU, CR

Abbreviations: Hb, hemoglobin; HU, hydroxyurea; BU, busulfan; SRT, splenic irradiation; CR, complete hematologic response; PR, partial hematologic response; BMT, bone marrow transplantation; NA, not available; ND, not determined; NAP, Neutrophil alkaline phosphatase (normal range, 30 to 100 units); Bl, blasts; Pm, promyelocytes; My, myelocytes; Met, metamyelocytes; Neut, neutrophil; Eos, eosinophil; Baso, basophil; Mono, monocyte; Lymph, lymphocyte; NRBC, nucleated RBC.

*Spleen size was measured in cm below left costal margin.

†Two patients had blood films not typical of Ph^1-positive CML.

DNA was prepared by standard procedures. All samples of DNA were digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, and *Hind*III, separated on a 0.8% agarose gel by electrophoresis, and transferred to Hybond-N (Amersham, Buckinghamshire, UK) by the Southern technique. The filters were prehybridized and then hybridized[9] at 43 °C with radiolabeled (^{32}P) probe. The filters were washed twice for 60 minutes in 0.1 × SSC (sodium chloride and sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate at 43 °C and autoradiographed for three to five days at −70 °C.

The 0.6-kb intron fragment (*bcr*-G) was subcloned from a commercially available *bcr* probe (Oncogene Sciences, Mineola, NY) and was labeled by the oligonucleotide priming method to a specific activity of 1 to 3 × 10^9 cpm/μg (Fig 1).[10]

RESULTS

The hematologic findings for the seven patients are shown in Table 2. Their leukocyte counts ranged from 46 to 506 × 10^9/L at diagnosis. The neutrophil alkaline phosphatase

Table 2. Cytogenetic Findings in the Seven Patients

Patient	Source of Cells	Number of Metaphases	Karyotype
1	PB	38	46, XY
		2	46, XY, del (22)(pter→q11:)
2	BM	30	46, XY del (16)(pter→q22:)
3	BM*	30	46, XY
4	PB	20	46, XX
5	BM*	20	46, XY
6	PB	25	46, XX
7	BM	30	46, XX, t(4;9;22) (9pter→9q34::22q11→22qter; 22pter→22q11::4p11→4pter)

*Except in two cases peripheral blood (PB) or bone marrow (BM) for these studies was collected before any treatment was administered. Marrow cells from patients 3 and 5 were studied after the patients had been treated with chemotherapy and splenic irradiation, respectively.

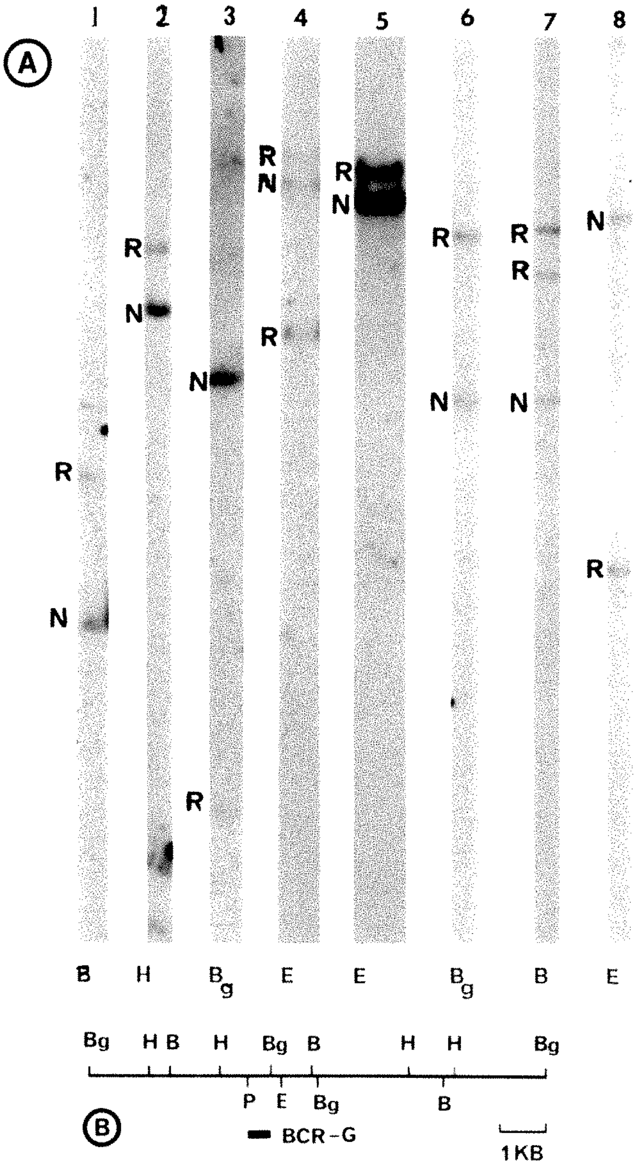

Fig 1. (A) Composite of Southern blots from the seven patients showing patterns of rearrangement for each patient. Lanes 1 to 7 show blots from each patient using DNA digested with one of the restriction endonucleases that showed rearrangement. The numbers correspond to the numbers given to each patient in Table 1. Lane 8 contains DNA from a patient with Ph^1-positive CML digested with *Eco*RI and hybridized with the bcr-G probe. The enzyme in each case is indicated below the corresponding lane (E = *Eco*RI, B = *Bam*HI, Bg = *Bgl*II, H = *Hind*III, N = Normal band, R = Rearranged band). (B) Schematic representation of the *bcr* region (3) showing the position of the probe (bcr-G) used.

score was low in the five patients studied at diagnosis. Two of the patients were thrombocytopenic. In each case the bone marrow in aspirate smears, trephine sections, or in both was hypercellular and showed gross granulocytic hyperplasia with maturation and no excess of blast cells. On morphologic grounds five patients (nos. 1, 2, 3, 6, and 7) were regarded as having a leukemia indistinguishable from Ph^1-positive CML. In two cases, however, the hematologic features were inconsistent with Ph^1-positive CML. Patient 4 had many hypogranular neutrophils, a high percentage and a high absolute number of monocytes, and a low basophil count; patient 5 also had gross morphologic abnormalities in the granulocyte series, including hypogranular or agranular myelocytes, metamyelocytes, and neutrophils, and he also lacked basophilia. In five of the seven cases, treatment usually effective in Ph^1-positive CML was effective in restoring the patients' blood counts to normal; in two cases (nos. 1 and 2) only partial responses were observed. All seven patients were alive at the time of writing. Two patients (nos. 3 and 4) had been treated by allogeneic bone marrow transplantation and were well 2 and 8 months posttransplant, respectively.

The mean number of CFU-GM for the five patients studied was 1,091 ± 752 (SEM) per 10^6 nucleated cells plated (range, 324 to 4,100) (Table 1). The corresponding mean values for ten patients with untreated Ph^1-positive CML and for five normal subjects were 947 ± 171 (range, 298 to 2,114) and 9 ± 3 (range, 2 to 19), respectively.

The results of cytogenetic studies are summarized in Table 2. Patient 7 had a complex translocation, and her karyotype may be regarded as showing a masked Ph^1 chromosome. Patient 2 had a deletion involving chromosome 16, but the karyotype was otherwise normal. A third patient (no. 1) had a minority population of myeloid metaphases that showed 22q− (but no 9q+), but the majority of metaphases were normal. The other four patients had entirely normal karyotypes.

Southern blots prepared from leukemic cells and probed with the *bcr*-G probe showed a germline together with an abnormal restriction band in digests with one or more restriction enzymes in all seven cases (Table 3, Fig 1). Germline bands only were seen in DNA from the blood of seven normal subjects (data not shown).

DISCUSSION

The definition of Ph^1-negative CML is necessarily imprecise because there is no single feature on which the diagnosis can be firmly based. In general, Ph^1-negative CML patients differ from Ph^1-positive patients in having a smaller spleen at corresponding leukocyte counts, more commonly thrombocytopenia at presentation, and a leukocyte differential that may lack a peak of myelocytes, may lack basophilia or eosinophilia, or may show monocytosis. There may also be dysplastic changes in the granulocyte series, which are rarely or never seen in Ph^1-positive disease. Five of the patients reported in this paper had a form of CML that was identical with Ph^1-positive disease, but in two (nos. 4 and 5), some features were atypical. In the five patients whose blood was cultured, the numbers of CFU-GM were in the range seen in Ph^1-positive CML and very different from those seen in chronic myelomonocytic leukemia or in normal subjects. Four of the seven patients had normal karyotypes. The three other patients had various chromosomal abnormalities, but none had the classic t(9;22).

Table 3. Schematic Representation of *bcr* Gene Rearrangements in the Seven Patients

	Rearrangement Detected With Restriction Enzyme			
Patient	*Bam*HI	*Bgl*II	*Eco*RI	*Hind*III
1	+	+	+	−
2	−	−	−	+
3	−	+	−	−
4	+	−	+	+
5	−	−	+	−
6	−	+	+	−
7	+	+	+	−

In one recent retrospective analysis of the hematologic features of 25 patients previously regarded as having Ph^1-negative CML, the authors concluded that all but one case could be better reclassified either as examples of the myelodysplastic syndrome or as chronic myeloproliferative disorders other than CML.[11] Essentially the same conclusion was reached in a retrospective study of 24 patients seen at the Mayo Clinic; the authors doubted the existence of Ph^1-negative CML as a real entity.[12] We believe, however, that Ph^1-negative CML is a heterogeneous group within which a minority of cases are indistinguishable on clinical and hematologic criteria from Ph^1-positive disease.

We found evidence of rearrangement in the *bcr* region in all seven patients studied in this series. These results appear to contrast with those of Morris et al who reported evidence for *bcr* rearrangement in only two of their five Ph^1-negative patients.[13] However, the three patients in that series without detectable *bcr* rearrangement were reclassified as having other forms of myeloproliferative disease. It is worth noting that we have also studied three other patients, not included in this series, with hematologic features consistent with chronic myelomonocytic leukemia; none had evidence of *bcr* rearrangement. In another recent report *bcr* rearrangement was found only in one of ten Ph^1-negative patients[14]; results of using only one restriction enzyme were presented, and different results might have been obtained if more enzymes had been used.

We cannot at present say whether the c-*abl* gene has come into juxtaposition with any portion of the *bcr* gene in these Ph^1-negative cases, and we do not know whether the rearranged *bcr* gene is or is not retained on chromosome 22. *abl*-related genetic material was demonstrated on chromosome 22 in one of the cases reported by Morris et al.[13] Moreover, Bartram and his colleagues[15] described a patient with clinically typical CML who had a complex chromosomal translocation, t(9;12;22); cytogenetic and molecular biologic studies suggested that the *bcr-abl* hybrid gene was present but was located on chromosome 12 rather than on chromosome 22 as in classic Ph^1-positive CML. However, in another case of Ph^1-negative CML, *bcr* rearrangement was demonstrated without apparent translocation involving c-*abl* or expression of the novel *abl*-related mRNA species.[16] Thus, the extent to which c-*abl* is involved with the *bcr* rearrangement in Ph^1-negative CML is not yet clear.

In conclusion, it seems that involvement of the *bcr* gene is a characteristic feature of CML, whether or not the karyotype is abnormal. It is clear, however, that clinically and hematologically the Ph^1-negative cases form a heterogeneous group, and the full details of the molecular basis for the variation remain to be worked out.

REFERENCES

1. de Klein A, Geurts van Kessel A, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR: A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 300:765, 1982

2. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, van Kessel AG, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, Heisterkemp N, Stephenson JR, Groffen J: Translocation of *c-abl* oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 306:277, 1983

3. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region—*bcr*—on chromosome 22. Cell 36:93, 1984

4. Shtivelman E, Lipshitz B, Gale RP, Canaani E: Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukaemia. Nature 315:550, 1985

5. Cannani E, Gale RP, Steiner-Saltz D, Berrebi A, Aghai E, Januszewicz E: Altered transcription of an oncogene in chronic myeloid leukaemia. Lancet 1:593, 1984

6. Konopka JB, Watanabe SM, Singer JW, Collins SJ, Witte ON: Cell lines and clinical isolates derived from Ph^1-positive chronic myelogenous leukemia patients express *c-abl* proteins with a common structural alteration. Proc Natl Acad Sci USA 82:1810, 1985

7. Hellmann A, Th'ng KH, Goldman JM: Production of colony stimulating activity in mixed mononuclear cell culture. Br J Haematol 45:245, 1980

8. Seabright M: A rapid banding technique for human chromosomes. Lancet 2:971, 1983

9. Anderson MLM, Szajnert MF, Kaplan JC, McColl L, Young BD: The isolation of a human IgV$_\lambda$ from a recombinant library of chromosome 22 and estimation of its copy number. Nucleic Acids Res 12:6647, 1984

10. Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6, 1983

11. Pugh WC, Pearson M, Vardiman JW, Rowley JD: Philadelphia chromosome–negative chronic myelogenous leukaemia: A morphological reassessment. Br J Haematol 60:457, 1985

12. Travis LB, Pierre RV, DeWald GW: Ph^1-negative chronic granulocytic leukemia: A nonentity. Am J Clin Pathol 85:186, 1986

13. Morris CM, Reeve AE, Fitzgerald PH, Hollings PE, Beard MEJ, Heaton DC: Genomic diversity correlates with clinical variation in Ph^1-negative chronic myeloid leukaemia. Nature 320:281, 1986

14. Bartram CR, Carbonell F: *bcr* rearrangement in Ph-negative CML. Cancer Genet Cytogenet 21:183, 1986 (letter)

15. Bartram CR, Kleihauer E, de Klein A, Grosveld G, Teyssier JR, Heisterkamp N, Groffen J: *c-abl* and *bcr* are rearranged in a Ph^1-negative CML patient. EMBO J 4:683, 1985

16. Bartram CR: *bcr* rearrangement without juxtaposition of *c-abl* in chronic myelocytic leukemia. J Exp Med 162:2175, 1985

CONCISE REPORT

A De Novo Intragenic Deletion of the Potential EGF Domain of the Factor IX Gene in a Family With Severe Hemophilia B

By M. Vidaud, C. Chabret, C. Gazengel, L. Grunebaum, J.P. Cazenave, and M. Goossens

We have studied a family of three patients who were severely afflicted with hemophilia B without inhibitor for their factor IX genes through the use of factor IX cDNA and genomic DNA probes. The patients had detectable (30% of normal) factor IX antigen. DNA hybridization analysis demonstrated that these patients had a partial intragenic deletion in their factor IX gene. This 2.8-kb deletion included exon d and the surrounding sequences. This exon codes for the amino acid sequence from No. 47 through 84 of the factor IX protein and contains its first potential EGF domain; the de novo occurrence of the mutation in the grandfather's germ cells was established by linkage analysis. This specific gene has been named F IX$^{\text{Strasbourg}}$.

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FACTOR IX is the precursor of a serine protease required for blood clotting by the intrinsic coagulation pathway.[1] Defects in this factor are inherited as an X-linked trait and result in hemophilia B (or Christmas disease), a heterogeneous bleeding disorder that occurs in about one in 30,000 newborn males.[2] This heterogeneity, observed at the clinical and biochemical levels, probably reflects the existence of a large collection of different genetic lesions. In addition, as for hemophilia A, new mutations continually arise in various populations. Previous molecular screening studies using factor IX gene probes have described a series of gross gene deletions, mainly associated with the presence of inhibitors[3-6] and point mutations.[7]

We report a family in which a woman gave birth to three male children having severe hemophilia B without inhibitor. At the request of the family for a prenatal diagnosis, a study was undertaken and an intragenic deletion was found and mapped in the factor IX gene. This deletion involved exon d, a coding sequence that forms part of the connecting peptide region and the first potential growth factor domain of the protein.[8-10] The de novo occurrence of the mutation was established, and linkage analysis of DNA polymorphisms in other family members revealed that this new mutation had taken place in the germ cells of the maternal grandfather.

MATERIALS AND METHODS

The probands, three boys with hemophilia B, had less than 0.01 U/mL factor IX clotting activity (determined by the one-stage assay of F IX:C) and 0.3 U/mL factor IX:antigen measured by an immunoenzymatic method (Asserachrom FIX:Ag, Stago, Paris) 2 weeks after transfusion, in several instances. The factor IX:antigen was found to be the same in each affected member. The three boys had severe disease with frequent (an average of two per month) hemarthrosis and repeated hemorrhages. Nuclear DNA was isolated from leukocytes in 10 to 20 mL of EDTA-anticoagulated blood or from chorionic cells as described previously,[11] and digested with *Taq*I, *Eco*RI, *Hind*III, *Bam*HI, *Bgl*II, and *Pvu*II restriction endonucleases under conditions recommended by the commercial suppliers. Southern blotting was carried out as described elsewhere.[12,13] The cDNA fragment cVII and the subgenomic probe VIII[8] were kindly provided by Professor G. G. Brownlee, Oxford University, Oxford, England.

RESULTS

The family (see Fig 1) was referred to us for prenatal diagnosis of hemophilia B, when the carrier mother (III-1), who already had three affected boys (IV-1, IV-2, nonidentical twins, and IV-3), was nine weeks pregnant. No DNA studies had been performed before. The DNA samples were first analyzed with *Taq*I, an enzyme that permits detection of a polymorphic site that maps adjacent to exon d of the factor IX gene. This restriction fragment-length polymorphism (RFLP) can easily be identified with use of probe VIII.[14,15] The length of the two polymorphic fragments is 1.8 kb when the polymorphic site is absent and 1.3 kb when this site is present. Unexpectedly, instead of the 1.8- or 1.3-kb polymorphic fragment and the 5.3-kb invariable fragment, the three hemophiliacs had a single 7.0-kb fragment. Their mother (III-1) had three hybridizing fragments measuring 7.0, 5.3, and 1.3 kb. The fetal DNA (subject IV-4) displayed the 5.3- and 1.3-kb band, and in comparison with the DNA patterns observed in the mother and the affected boys, this was diagnostic of a normal factor IX gene.[16]

Figure 1A shows the pedigree of the family in which the mother (III-1), an obligate carrier, can be seen to have inherited the 1.3-kb *Taq*I polymorphic fragment from her mother (II-1), who is homozygous for this fragment. The grandfather (II-2) is hemizygous for the 1.8-kb fragment, and his daughters (III-3, III-4, III-5, and III-6), except the mother of the hemophiliacs, have inherited the two markers: the 1.3-kb allele from their mother and the 1.8-kb allele from their father. This analysis of the DNA polymorphisms revealed that the mutation to the hemophilia B gene occurred between generations II and III, in germ cells of subject II-2.

In order to further explore the abnormality revealed by *Taq*I analysis, DNA samples from a normal male, from the carrier, and from the hemophiliacs were analyzed with a variety of restriction endonucleases (Table 1), using probes VIII and cVII. Probe VIII is a *Hind*III/*Eco*RI genomic fragment that contains exon d and 1.7 kb of the adjacent 5′

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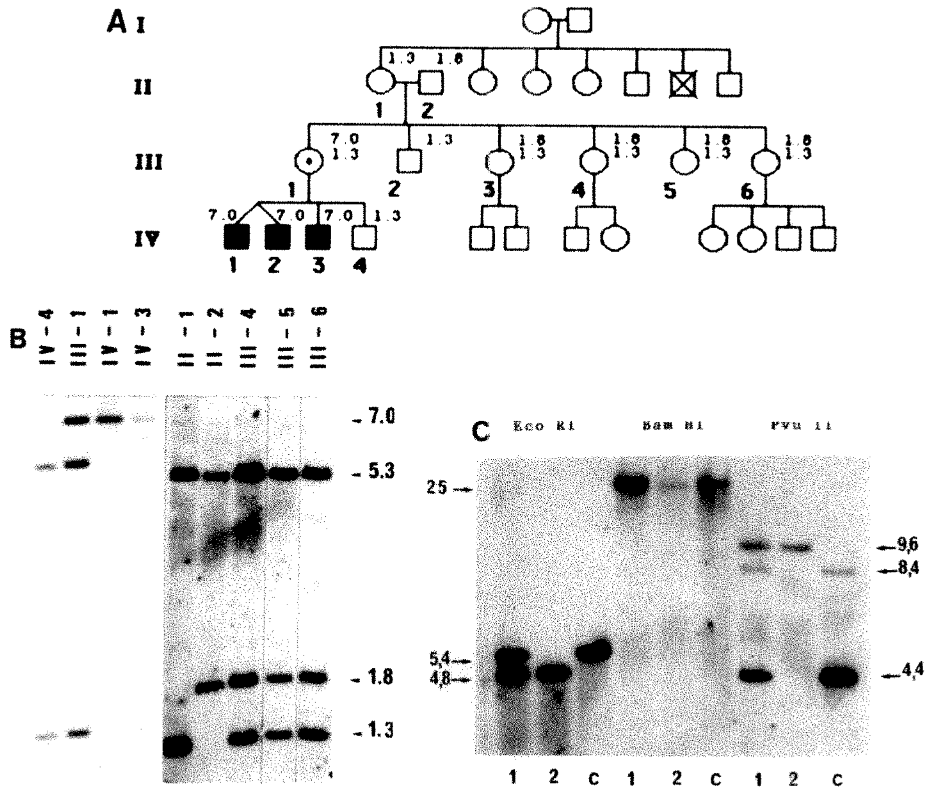

Fig 1. (A) Pedigree of the factor IX-deficient family. *TaqI* RFLP type is at the top of subject's symbol when DNA has been analyzed. ⊙, carrier female; ■, affected; □, normal. (B) Southern blot analyses of DNAs digested with *TaqI* and hybridized with the genomic probe VIII from patients, obligate carrier, and normal persons of the family. The size of DNA fragments is expressed in kilobases. (C) Southern blot analyses of 1, the carrier mother (III-1); 2, an affected boy (IV-1); and C, a normal male. Filters were hybridized with probe VIII.

intron. Its use allowed us to demonstrate that the mutation is a deletion and to map the border of the deleted region by comparative evaluation of the length of the normal fragments and of the truncated or fused fragments found in the abnormal DNA (Fig 1C). Probe cVII, a sequence complementary to factor IX mRNA, permitted us to demonstrate that the other exon-containing restriction fragments are present (data not shown). The deletion involves 2.8 kb of DNA: the exon d and the surrounding flanking regions, as shown in Fig 2.

Table 1. Restriction Endonuclease Band Patterns Obtained After Blot Hybridization of DNA From the Mother and a Hemophiliac

	Subjects		
	III-1	IV-1	Normal
TaqI	7.0	7.0	
	5.3		5.3
	1.3		1.3 (1.8)
EcoRI	5.4		5.4
	4.8	4.8	
BamHI	~25	~25	~25
PvuII	9.6	9.6	
	8.4		8.4
	4.4		4.4
HindIII	4.6		4.6
	1.8	1.8	
BglII	7.8		7.8
	5.2	5.2	

DISCUSSION

The patients described in this paper have virtually no factor IX:C clotting activity, but detectable factor IX antigen. They can be classified as CRM-reduced patients with antigen in excess of clotting activity. This status raises the possibility that an abnormal factor IX molecule with no clotting activity is produced in decreased amount.[17] Our results indicate that exon d and its junctions with the two flanking introns are missing in the defective gene. This exon specifies 38 amino acids of the EGF-like growth factor domain of the protein, and the unique β-hydroxyaspartic acid occurs at position 64 in this region. Giannelli et al[3] have previously reported patients with gross deletions of the factor IX gene, and Hassan et al[5] recently described a deletion involving ~33 kb of the gene. The patients described in these studies had detectable antibodies against the factor IX protein. More recently, Chen et al[6] have shown that in a family, several subjects have an intragenic deletion, involving exons e and f of the factor IX gene, without detectable inhibitory antibodies in their sera.

The deletional mutation reported in this paper is also located in the middle portion of the gene and is limited to a loss of 2.8 kb of DNA containing exon d. We have named this mutant F $\text{IX}^{\text{Strasbourg}}$. It is likely that the defective gene is in some manner transcribed and translated. We can postulate that exons c and e are linked in the abnormal mature messenger RNA, giving rise to a shorter factor IX protein.

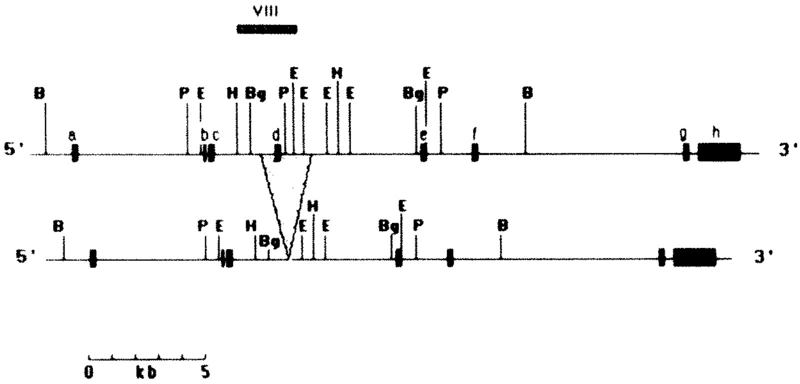

Fig 2. The site of the deletion in the abnormal factor IX gene with *Eco*RI (E), *Hind*III (H), *Bgl*II (Bg), *Bam*HI (B), *Pvu*II (P) sites. The organization of the normal gene is taken from Anson et al.[8] Exons are shown as solid regions labeled from a through h. The site of the genomic probe VIII is depicted as a bar. The lower part of the figure shows the abnormal factor IX gene. Only the restriction sites useful to mapping of the deletion are represented.

The deletion of exon d and its surrounding sequences would produce a hybrid intron having the donor site of the intron located 5′ to the deleted DNA fragment and the acceptor site of the next one. This hybrid intron could thus be spliced out. The intermediate amount of factor IX antigen might be the consequence of either an instability of the structurally abnormal protein or an inefficiency of the mRNA maturation process.

Another observation made in this study is the de novo occurrence of the mutation. According to the Haldane hypothesis,[18] newly arising mutations account for up to one third of hemophilia families. In these families, DNA analysis can provide reassurance to female relatives about carrier status and give additional information about sex difference in the mutation rate for hemophilia. In the family presented here, the mutation must have originated in the grandfather's germ cells, although the possibility of false paternity of the carrier (III-1) cannot be excluded. The sisters of the obligate carrier woman can be classified as noncarriers on the basis of the segregation pattern observed in DNA analyses. In this family, the cDNA probe was successfully used for carrier detection, genetic counseling, and prenatal diagnosis.

This small deletion was detected because it lies in part of the gene that is complementary to the cDNA probe used to analyze the *Taq*I polymorphic marker. It is likely that many similar deletions may go undetected due in part to the large size of the factor IX gene, unless they are specifically searched for, using a set of DNA probes spanning the entire gene. The size of the gene and the extent of heterogeneity of the mutations may render difficult the study of molecular defects of the factor IX gene. The identification of defective genes will be facilitated by the use of DNA polymorphisms, although, unfortunately, in this gene only four high-frequency polymorphic sites have been discovered to date.

REFERENCES

1. Hedner U, Davie EW: Factor IX, in Colman RW, Hirsh J, Marder VJ, Salzman EW (eds): Hemostasis and Thrombosis. Philadelphia, JB Lippincott, 1982, p 29

2. McKee PA: Hemostasis and disorders of blood coagulation, in Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds): The Metabolic Basis of Inherited Disease (ed 5). New York, McGraw-Hill, 1983, p 1531

3. Giannelli F, Choo KH, Rees DJG, Boyd Y, Rizza CR, Brownlee GG: Gene deletion in patients with hemophilia B and antifactor IX antibodies. Nature 303:181, 1983

4. Peake IR, Furlong BL, Bloom AL: Carrier detection by direct gene analysis in a family with haemophilia B (factor IX deficiency). Lancet 1:242, 1984

5. Hassan HJ, Leonardi A, Guerriero R, Chelucci C, Cianetti L, Ciavarella N, Ranieri P, Pilolli D, Peschle C: Hemophilia B with inhibitor: Molecular analysis of the subtotal deletion of the factor IX gene. Blood 66:728, 1985

6. Chen SH, Yoshitake S, Chance PF, Bray GL, Thompson AR, Scott CR, Kurachi K: An intragenic deletion of the factor IX gene in a family with hemophilia B. J Clin Invest 76:2161, 1985

7. Rees DJG, Rizza CR, Brownlee GG: Haemophilia B caused by a point mutation in a donor splice junction of the human factor IX gene. Nature 316:643, 1985

8. Anson DS, Choo KH, Rees DJG, Giannelli F, Gould K, Huddleston JA, Brownlee GG: The gene structure of human antihaemophilic factor IX. EMBO J 3:1053, 1984

9. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K: Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). Biochemistry 24:3736, 1985

10. Rogers J: Exon shuffling and intron insertion in serine protease genes. Nature 315:458, 1985

11. Goossens M, Dumez L, Kaplan L, Lupker M, Chabret C, Henrion R, Rosa J: Prenatal diagnosis of sickle-cell anemia in the first trimester of pregnancy. N Engl J Med 309:831, 1983

12. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

13. Goossens M, Kan YW: DNA analysis of the diagnosis of hemoglobin disorders, in Antonini E, Rossi-Bernardi L, Chiancone E (eds): Methods in Enzymology, vol 76. Orlando, Fla, Academic Press, 1981, p 805

14. Giannelli F, Choo KH, Winship PR, Anson DS, Rees DJG, Ferrari N, Rizza CR, Brownless GG: Characterisation and use of an intragenic polymorphic marker for detection of carriers of haemophilia B (factor IX deficiency). Lancet 1:239, 1984

15. Winship PR, Anson DS, Rizza CR, Brownlee GG: Carrier detection in haemophilia B using two further intragenic fragment length polymorphisms. Nucleic Acids Res 12:8861, 1984

16. Goossens M, Daffos F, Forestier F, Gazengel C: First trimester prenatal diagnosis of severe Christmas disease. Thromb Haemost 54:01624, 1985 (abstr)

17. Bertina RM, Van der Linden JK: Detection and classification of molecular variants of factor IX, in Bloom AL (ed): Methods in Hematology, vol 5. New York, Churchill Livingstone, 1982, p 137

18. Haldane JBS: The rate of spontaneous mutation of a human gene. J Genet 31:317, 1935

CONCISE REPORT

The Spectrum of β-Thalassemia Genes in China and Southeast Asia

By Haig H. Kazazian, Jr., Carol E. Dowling, Pamela G. Waber, Shangzhi Huang, and Wilson H.Y. Lo

To make possible prenatal diagnosis of β-thalassemia in China and Southeast Asia by direct detection of mutant β-globin genes, we have determined the spectrum of mutations producing the disorder in this region of the world. Seventy-eight β-thalassemia genes from Chinese and Southeast Asians were randomly obtained, and the relevant mutation was characterized in 76 (98%) of them. Seven different point mutations were found among the 78 genes studied. Of these seven β-thalassemia alleles, two constitute 62%, and two others account for 29% of the total. Since only four alleles make up 91% of the mutant genes, prenatal diagnosis of β-thalassemia in China and Southeast Asia should be feasible by simplified techniques for direct detection of point mutations.

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β-THALASSEMIA (β-thal) is a severe disorder of β-globin gene expression characterized by hypochromic, hemolytic anemia and inherited as an autosomal disorder.[1,2] The disease has a high frequency in regions of the world endemic for malaria, including the Mediterranean Basin, Africa, India, South China, and Southeast Asia. To date, 37 different point mutations producing β-thal have been characterized, and in general each ethnic group appears to carry its own set of mutant alleles.[3-6] Because life expectancy for affected individuals carrying two defective β-globin genes is usually 25 years or less even with transfusion therapy,[2] prenatal diagnosis has been an important option for couples in which both members are carriers of a β-thal gene.[7-9] These diagnoses have often been carried out by globin chain synthesis studies[9] or DNA polymorphism analyses.[10] Direct detection of the mutant gene(s) involved has simplified prenatal diagnosis in Sardinia, where nearly all cases of β-thal are due to a single mutation, nonsense codon 39.[11]

To understand the heterogeneity of β-thal mutations and make possible simplified prenatal diagnosis of the disorder in China and Southeast Asia, we carried out oligonucleotide hybridization analysis on 78 β-thal alleles. Seven point mutations in the β-globin gene account for 98% of the β-thal genes. Therefore, prenatal diagnosis can be provided by direct detection of the molecular defect using a small number of oligonucleotides.

MATERIALS AND METHODS

Our subjects were β-thal patients and their parents from the cities of Guangzhou and Zhangjiang of Kwantung province and Chinese and Southeast Asian families residing in the United States and Canada referred for prenatal diagnosis. DNA was extracted from the leukocytes of EDTA-anticoagulated blood samples and subjected to analysis of DNA polymorphisms in the β-globin gene cluster as described.[12] Oligonucleotides (19-mers) were synthesized on a DNA synthesizer (Applied Biosystems, Foster City, Calif). These oligonucleotides were specific for β-thal mutations at −29, −28, codon 17, codons 41-42, codons 71-72, IVS-1 nt 5, and IVS-2 nt 654. Oligomers specific for the normal β-globin gene sequence in these regions were also synthesized. Ten-microgram samples of genomic DNA of various subjects were subjected to oligonucleotide hybridization analysis with various oligonucleotides as described.[13,14]

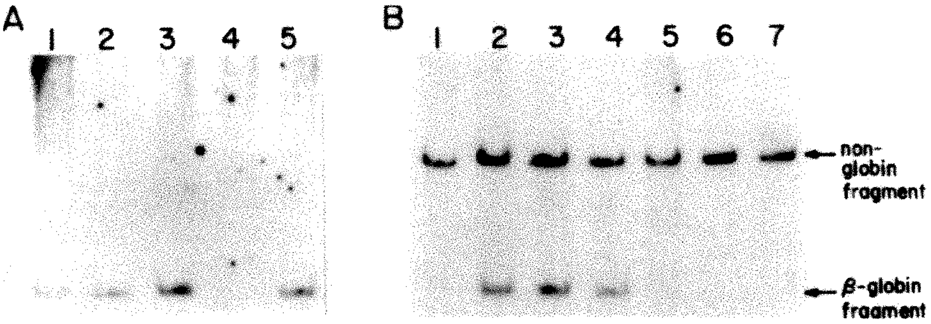

Fig 1. Oligonucleotide hybridization with probes specific for mutant β-thal alleles. Ten micrograms of genomic DNA from various individuals heterozygous for β-thal was digested with *Bam*HI and subjected to electrophoresis in an agarose gel, and the dried gel was hybridized with specific oligonucleotides (19-mers). Hybridization and wash conditions were as described.[13,14] In panel A, the probe (GGTTGAGTCCTTTGGGGAT) is specific for the frameshift 41-42 mutation, and in panel B the probe (GGCATAGAAGTCAGGGCAG) is specific for the −28 G-A mutation. Individuals in lanes 1 to 3, five in panel A, and two to four in panel B are positive for the tested mutant allele, whereas the remaining individuals in both panels are negative.

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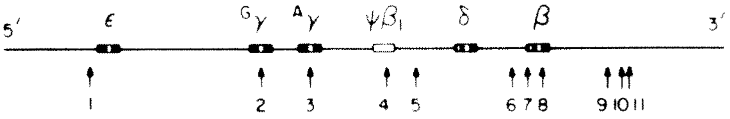

Table 1. Haplotypes of β-Thal Chromosomes in China and Southeast Asia

	5′ ε	G_γ	A_γ	$\psi\beta_1$		δ β	3′	
Haplotype	↑ 1	↑ 2	↑ 3	↑ 4	↑ 5	↑ ↑ ↑ 6 7 8	↑ ↑ ↑ 9 10 11	No. of Chromosomes
1a	+	−	−	−	−	+ + +	+ − +	31
1b	+	−	−	−	−	− + +	+ − +	5
2	+	−	−	−	−	+ − −	+ − +	25
3	−	+	+	−	+	+ − −	+ − +	12
4	+	−	−	−	−	+ + +	+ + +	1
5	−	+	−	+	+	+ − −	+ − +	1
6	+	−	−	−	−	+ + +	+ + −	3
Total								78

Haplotypes were constructed by family studies as previously reported.[12] The DNA polymorphisms examined are (1) *Hin*cII, 5′ to the ε-globin gene; (2) *Hin*dIII, $^G\gamma$-globin gene IVS-2; (3) *Hin*dIII, $^A\gamma$-globin gene IVS-2; (4) *Hin*cII, $\psi\beta_1$-globin gene; (5) *Hin*cII, 3′ to the $\psi\beta_1$-globin gene; (6) *Hin*fI, 5′ to the β-globin gene; (7) *Hgi*AI, β-globin gene; (8) *Ava*II, β-globin gene IVS-2; (9) *Hpa*I, 3′ to the β-globin gene; (10) *Hin*dIII, 3′ to the β-globin gene; (11) *Bam*Hi, 3′ to the β-globin gene.

RESULTS

Seventy-eight β-thal genes in Chinese and Southeast Asians were available for study. Of these, 36 were from patients residing in South China, and 35 were from Chinese living in the United States and Canada. Seven genes were from Southeast Asians: four from Vietnamese and three from Laotians.

These genes were subjected to haplotype analysis,[12] and based on this analysis, seven of these β-globin genes were cloned and sequenced.[6,15-17] An eighth gene had been subjected to mRNA sequencing by Chang and Kan.[18] These sequence analyses had previously demonstrated the presence of seven alleles in China and Southeast Asia. For this study we synthesized oligonucleotides specific for these seven alleles and determined their frequency among the 78 genes available for study. An example of oligonucleotide analysis with two mutant probes in shown in Fig 1. These seven mutations accounted for 76 of the 78 β-thal genes; two uncharacterized β-thal genes remain in our sample after oligonucleotide analyses with a number of mutant probes.

β-thal genes were present in the six haplotypes of Table 1.[19] Haplotype 1 is divided into 1a and 1b based on the presence or absence of the polymorphic *Hin*fI site 5′ to the β-globin gene. We have reported a number of examples of variation in this *Hin*fI site within a specific β-thal haplotype associated with a specific β-thal mutation.[5] In this study both alleles found in haplotype 1, IVS-1 nt 654 and frameshift 41-42, were present in haplotypes 1a and 1b. Haplotypes 1, 2, and 3 have frequencies similar to those previously reported.[6,19] Haplotypes 4, 5, and 6 were rarely observed.

As in Mediterranean and Indian β-thal, there exists a close, but not invariant, association of each haplotype with a specific β-globin mutation (Table 2). The IVS-2 nt 654 allele was observed only in haplotype 1, and all but one instance of the nonsense 17 allele was found in association with haplotype 2. The −28 mutation was seen most commonly in

Table 2. Association of β-Thal Mutations and Chromosomal Backgrounds in Chinese and Southeast Asians

		Mutations							
β-Gene* Framework	Haplotype	IVS-2 nt 654	Frameshift 41-42	Nonsense 17	−28 A-G	−29 A-G	IVS-1 nt 5	Frameshift 71-72	Unknown
	1a	19	12						
1	1b	1	4						
	4						1		
2	6					3			
	2		12	9	2			1	1
3a	3			1†	11				
	5								1†
	Total	20 (26%)	28 (36%)	10 (13%)	13 (16%)	3 (4%)	1	1	2

*β-globin gene frameworks are distinguished by the presence or absence of five nucleotide polymorphisms in the β-globin gene, four of which are in intron 2 and one is in exon 1. The most common form of the polymorphism is present at all five sites in framework 1, four sites in framework 2, and one site in framework 3a.[3]

†These genes are present in a β°-thal patient[18] who has β-gene haplotypes 3 and 5. Since DNA on family members has not yet been available to us, either the uncharacterized allele or the nonsense 17 allele could be associated with haplotype 5 and the other with haplotype 3 in this patient.

haplotype 3. Only the very common frameshift 41-42 allele was seen in two haplotypes, 1 and 2, which contain different β-globin gene frameworks, 1 and 3a. Finding this allele in different β-globin gene frameworks suggests that it has migrated to a new β-globin gene background by an interallelic gene conversion event.[3] In addition, this frameshift 41-42 allele accounts for six of the seven β-thal genes studied in Southeast Asians, the remaining β-thal gene being a nonsense 17 allele. Frameshift 71-72, previously speculated to be a common mutation in Chinese because of its discovery in a common haplotype,[6] was found to be rare in this population.

DISCUSSION

Our data suggest that β-thal has now been nearly completely characterized in this region of the world. We would expect that other mutations to β-thal exist in this region but that they are quite uncommon and have not yet attained significant frequencies in the population. Most importantly, the data suggest that four alleles account for up to 91% of β-thal genes in this region. In comparison, six different mutations account for 92% of β-thal genes in the Mediterranean Basin.[20] Direct detection of mutations, whether using specific oligonucleotides, RNA hybridization and RNAse I cleavage,[21] or some yet-to-be-discovered method, should be feasible for prenatal diagnosis since the number of common mutations is relatively small. Such direct detection of mutations by improved methods should simplify prenatal diagnosis of β-thal in this region.

In a province-by-province screening for β-thal in China, Yang et al found carrier frequencies of .018 to .055 in four provinces, Fukien, Kweichow, Szechuan, and Yunnan.[22] From the carrier frequencies observed and the population of each province, we estimate that 1,800 to 2,000 pregnancies per year are at 25% risk of β-thal in the fetus. Wide-scale screening for the β-thal trait followed by prenatal detection using direct analysis of the mutant genes in couples at risk should be highly feasible in the prevention of this disease in South China.

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REFERENCES

1. Bunn HF, Forget BG: Hemoglobin: Molecular, Genetic and Clinical Aspects. Philadelphia, Saunders, 1986

2. Weatherall DJ, Clegg JB: The Thalassaemia Syndromes. Boston, Blackwell, 1981

3. Orkin SH, Kazazian HH, Jr: The mutation and polymorphism of the human β-globin gene and its surrounding DNA. Annu Rev Genet 18:131, 1984

4. Antonarakis SE, Orkin SH, Cheng T-C, Scott AF, Sexton JP, Trusko S, Charache S, Kazazian HH Jr: β-thalassemia in American blacks: Novel mutations in the TATA box and IVS-2 acceptor splice site. Proc Natl Acad Sci USA 81:1154, 1984

5. Kazazian HH, Jr, Orkin SH, Antonarakis SE, Sexton JP, Boehm CD, Goff SC, Waber PG: Molecular characterization of seven β-thalassaemia mutations in Asian Indians. EMBO J 3:593, 1984

6. Cheng T, Orkin SH, Antonarakis SE, Potter MJ, Sexton JP, Giardina PJV, Li A, Kazazian HH Jr: β-thalassemia in Chinese: Use of in vivo RNA analysis and oligonucleotide hybridization in systematic characterization of molecular defects. Proc Natl Acad Sci USA 81:2821, 1984

7. Kan YW, Golbus MS, Klein P, Dozy A: Successful application of prenatal diagnosis in a pregnancy at risk for homozygous β-thalassemia. N Engl J Med 292:1096, 1975

8. Alter BP, Modell CB, Fairweather D, Hobbins JC, Mahoney MJ, Frigoletto FD, Sherman AS, Nathan DG: Prenatal diagnosis of hemoglobinopathies: A review of 15 cases. N Engl J Med 295:1437, 1976

9. Alter BP: Prenatal diagnosis of hemoglobinopathies: A status report. Lancet 2:1152, 1981

10. Boehm CD, Antonarakis SE, Phillips JA, III, Stetten G, Kazazian HH, Jr: Prenatal diagnosis using DNA polymorphisms: Report of 95 pregnancies at risk for sickle-cell or β-thalassemia. N Engl J Med 308:1052, 1983

11. Pirastu M, Kan YW, Cao A, Conner BJ, Teplitz RL, Wallace RB: Prenatal diagnosis of β-thalassemia: Detection of a single mutation in DNA. N Engl J Med 309:284, 1983

12. Antonarakis SE, Boehm CD, Giardina PJV, Kazazian HH, Jr: Nonrandom association of polymorphic restriction sites in the β-globin gene cluster. Proc Natl Acad Sci USA 79:137, 1982

13. Studencki AB, Wallace RB: Allele-specific hybridization using oligonucleotide probes of very high specific activity: Discrimination of the human β^{A}- and β^{S}-globin genes. DNA 3:7, 1984

14. Waber PG, Bender MA, Gelinas RE, Kattamis C, Karaklis A, Sofroniadou K, Stamatoyannopoulos G, Collins FS, Forget BG, Kazazian HH Jr: Concordance of a point mutation 5′ to the $^{A}\gamma$-globin gene with $^{A}\gamma\beta^{+}$ hereditary persistence of a fetal hemoglobin in Greeks. Blood 67:551, 1986

15. Orkin SH, Sexton JP, Cheng T-C, Goff SC, Giardina PJV, Lee JI, Kazazian HH Jr: ATA box transcription mutation in β-thalassemia. Nucleic Acids Res 11:4727, 1983

16. Kimura A, Matsunaga E, Takihara Y, Nakamura T, Takagi Y: Structural analysis of a β-thalassemia gene found in Taiwan. J Biol Chem 258:2748, 1983

17. Huang S-Z, Wong C, Antonarakis SE, Lo WHY, Kazazian HH, Jr: The same TATA box β-thalassemia mutation in Chinese and US Blacks: Another example of independent origins of mutation. Hum Genet (submitted for publication)

18. Chang JC, Kan YW: β^{0}-thalassemia, a nonsense mutation in man. Proc Natl Acad Sci USA 76:2886, 1979

19. Huang S-Z, Kazazian HH, Jr, Waber PG, Lo WHY, Ruolian C, Mei-qi, W: β-thalassemia in Chinese: Analysis of polymorphic restriction site haplotypes in the β-globin gene cluster. Chin Med J (Engl) 98:881, 1985

20. Kazazian HH, Jr, Orkin SH, Markham AF, Chapman CR, Youssoufian H, Waber PG: Quantification of the close association between DNA haplotypes and specific β-thalassaemia mutations in Mediterraneans. Nature 310:152, 1984

21. Myers RH, Larin Z, Maniatis T: Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 230:1242, 1985

22. Yang T-Y, Yang X-Y, Ch'en W-C, Qui S-L, Jin Y, Gan WJ, Qu Q: Thalassemia in China, in Bank A, Anderson WF, Zaino EC (eds): Fifth Cooley's Anemia Symposium. New York, New York Academy of Sciences, 1985, p 92

CONCISE REPORT

c-myc and *c-fos* Expression During Interferon-α Therapy for Hairy Cell Leukemia

By Pierre Lehn, François Sigaux, David Grausz, Pascale Loiseau, Sylvie Castaigne, Laurent Degos,
Georges Flandrin, and François Dautry

Low-dose interferon-α (IFN-α) therapy is consistently effective in the treatment of hairy cell leukemia (HCL). In two cases of resistance to IFN-α administration, we diagnosed variant HCL, a form of HCL with intermediate features between typical HCL and B cell prolymphocytic leukemia. We tried to distinguish variant and typical hairy cells (HCs) by Northern blot analysis of the oncogenes expressed in vivo. We report that variant HCs contain *c-myc* transcripts in contrast to typical HCs, whereas *c-fos* transcripts are detected in both cell types. We also report that the mRNA levels of *c-myc* are not modified in variant HCs by IFN-α treatment, whereas the level of *c-fos* mRNA is modulated in both types of HCs. Our findings suggest that the failure to modulate *c-myc* expression in vivo might indicate the limits of low-dose IFN-α therapy.

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HAIRY CELL LEUKEMIA (HCL) is a malignancy of a late stage of B cell differentiation[1] that can be effectively treated with low-dose interferon-α (IFN-α) therapy.[2-5] Variant HCL, a recently described rare form of HCL, appears as an intermediate disorder between typical HCL and B cell prolymphocytic leukemia.[6,7] We diagnosed two new cases of variant HCL (F. Sigaux and G. Flandrin, manuscript in preparation) that failed to respond to IFN-α. Because the mechanism of the antitumor activity of IFN-α in HCL is unknown, our observation offers a unique opportunity to investigate it.

A direct effect of IFN-α on the leukemic cells is one possible mechanism. Indeed, IFN-α administration leads, over the first months of treatment, to a progressive disappearance of the HCs without evidence of cell differentiation or marked effects on normal lymphocytic subpopulations.[8] Previous in vitro experiments with tumor cell lines have indicated that quiescent cells are particularly susceptible to growth inhibition by IFN.[9] This suggests a basis for the sensitivity of typical HCLs to low-dose IFN-α therapy because typical HCs have a very low level of proliferation in vivo.[10] Thus, we chose to compare the steady-state mRNA levels of *c-myc* in variant and typical HCs, given that *c-myc* expression is closely associated with cellular proliferation.[11] We extended our study to *c-fos*, which encodes another nuclear protein and whose expression seems to be restricted to differentiated cells with a low level of proliferation.[12,13]

MATERIALS AND METHODS

Hairy cell samples. After informed consent, human samples were obtained. We indicate in Table 1 the hematologic data of the patients before IFN-α therapy as well as the origin and the composition of the cell samples obtained by Ficoll-Hypaque density sedimentation. We restricted our study to the rare HCL patients with hyperleukocytosis (the two variant HCL cases and five out of more than 50 typical HCL patients included in our clinical trial evaluating the efficacy of IFN-α therapy) to ensure high levels of purity of the cell samples from the peripheral blood and to provide enough HCs for the Northern blot investigation. We also analyzed two splenic HCs samples obtained from patients undergoing splenectomy and not subjected to IFN-α therapy. Finally, we studied peripheral blood samples obtained during low-dose IFN-α therapy. Because we analyzed four typical HCL cases immediately after the first administration of IFN-α and because IFN-α therapy was ineffective in the two cases of variant HCL, neither the hematologic data nor the composition of the cell samples was significantly modified at the time of RNA extraction. The diagnosis of either typical or variant HCL was assessed by morphologic criteria. In all cases, HCs reacted with antibodies against HLA-DR, B cell–restricted antigens (B1 and B4), and with the anti-HC monoclonal antibody Leu-M5 (αS-HCL3).[14]

Northern blot analysis. Total cellular RNA was extracted by the guanidinium–cesium chloride method and analyzed by electrophoresis of 15 μg of RNA through 1.2% agarose-formaldehyde gels followed by Northern blot transfer to nitrocellulose and hybridization to the *onc* probes radiolabeled by nick translation.[15] Hybridization was performed at 68 °C in 4× SSPE (1× SSPE: 0.18 mol NaCl, 10 mmol sodium phosphate, pH 7.0, and 1 mmol Na$_2$ EDTA) containing 0.2% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate, and 5 μg/mL of heparin.[16] The filters were washed for one hour each in 1× SSPE + 0.1% SDS and 0.5× SSPE + 0.1% SDS at 68 °C before autoradiography. The *v-fos* probe was the *Bgl*II-*Pvu*II fragment of *v-fos*, and the *c-myc* probe was the *Cla*I-*Eco*RI fragment of the human *c-myc* gene containing the third exon. Several precautions were taken to ensure that RNA samples from the different patients could be compared. Ethidium bromide was used to stain each gel to confirm that equal amounts of RNA were loaded on the gel and to control the transfer of RNA from the gel to the nitrocellulose filter. The same control RNAs (HL60, K562, Ramos, and Molt4) were included on all blots and used as internal controls for determining the level of protooncogene expression by the

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Table 1. Initial Hematologic Data and Composition of Cell Samples

				Initial Blood Counts			Cell Samples			
Patients	Diagnosis	Splenectomy	Origin of Cells	WBC (10^9/dL)	HCs (%)	Monocytes (%)	HCs (%)	Monocytes (%)	Lymphocytes (%)	IFN-α*
1	Typical HCL	−	blood	18.3	72	0	97	0	3	1
2	Typical HCL	−	blood	17.3	58	0	90	0	10	2
3	Typical HCL	+	blood	59.5	85	3	98	0	2	2
4†	Typical HCL	+	blood	12.5	54	0	92	0	8	2
5	Typical HCL	+	blood	13.1	77	0	99	0	1	2
6	Typical HCL	+	spleen	—	—	—	99	0	1	—
7	Typical HCL	+	spleen	—	—	—	85	1	14	—
8	Variant HCL	+	blood	43.0	78	7	95	0	5	1
9	Variant HCL	−	blood	217.0	97	0	97	0	3	1

*IFN-α therapy: 1, 3 × 10^6 units daily of recombinant IFN-α_{2A} from Hoffmann-La Roche Inc, Basel, Switzerland; 2, 2 × 10^6 U/m^2 three times each week of recombinant INF-α_2 from Schering Corp, Kenilworth NJ.

†In case 4, the mononuclear cells were depleted of T lymphocytes by sheep erythrocyte rosetting.

HC samples. The blots were further standardized by hybridizing with a mouse β-actin probe.

RESULTS AND DISCUSSION

We could detect *c-myc* transcripts only in the variant HCs. These transcripts are of the normal size. Only in sample 9 is their level as high as that seen in the control cell lines K562, Ramos, and Molt4, but it remains lower than in the HL60 cell line (Fig 1B). Contrary to *c-myc*, normal-sized (2.2 kb) *c-fos* transcripts are present in HCs from all patients tested, although at variable levels (Fig 1A). The level of *c-fos* mRNA is typical HCs seems to depend on the presence or absence of the spleen (Fig 1A and Table 1). In addition (data not shown), we could not detect *c-myb* transcripts in both types of HCs, although they were present in two of the control cell lines, HL60 and Molt4. Harvey-ras mRNA accumulates at similar levels in the HCs and in the control cell lines; this is consistent with previous studies of Ha-ras gene expression in acute and chronic leukemias.[17] Finally, we could not detect *c-sis* messengers. This is of particular interest because of the relationship between *c-sis* protein and platelet-derived growth factor (PDGF) and the well-known myelofibrosis in HCL.

Our detection of *c-myc* transcripts exclusively in variant HCs suggests that variant HCL is a more proliferative disease then typical HCL. Because we could also detect

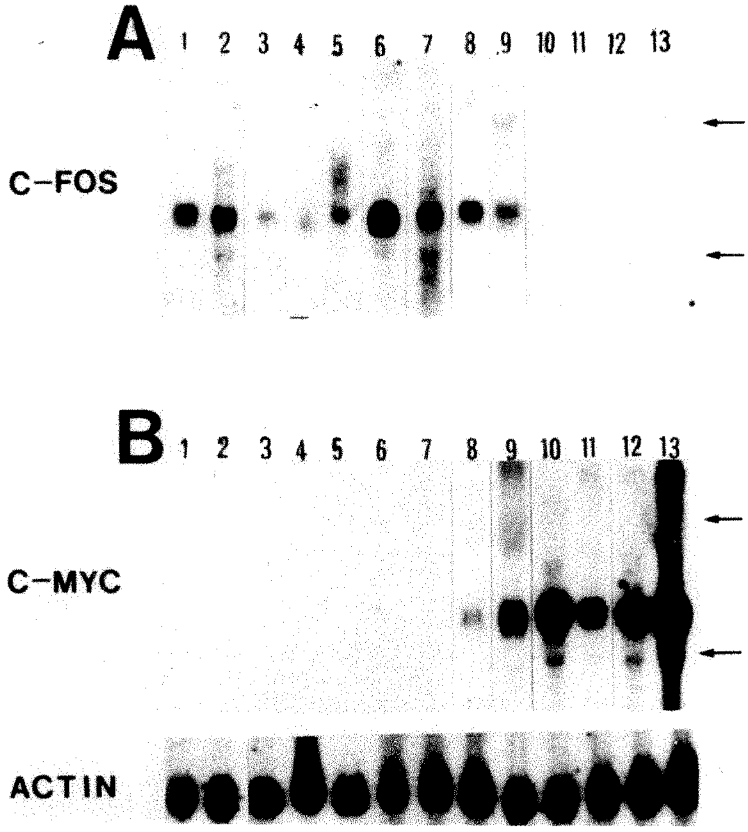

Fig. 1. Expression of *c-fos* (A) and *c-myc* (B) before IFN-α therapy. The RNA samples in lanes 1 to 9 are in an identical order with Table 1. RNA from the control cell lines Ramos, Molt 4, K562, and HL60 is shown in lanes 10 to 13, respectively. The arrows indicate the position of the ribosomal RNAs. The Figure also shows β-actin mRNA levels.

c-myc transcripts in two cases of B cell prolymphocytic leukemias (data not shown), our results strengthen the classification of variant HCL as an intermediate disorder between typical HCL and B cell prolymphocytic leukemia.[7] The heterogeneity of these rare intermediate diseases, already reflected in the difference in the *c-myc* mRNA levels in our two cases, could be further analyzed by the study of additional cases.

Our findings of *c-fos* expression in both typical and variant HCs could be related to their mature phenotype since a stable expression of *c-fos* has been reported only in some differentiated cells.[12] In the hematopoietic system in which a stable expression of *c-fos* is known in macrophages,[12,13] our results extend the domain of investigation to the B lymphocyte lineage. Recent observations indicate that *c-fos* transcripts can be detected during activation of normal human peripheral blood lymphocytes.[18,19] In addition we observed noticeable *c-fos* mRNA levels in prolymphocytic leukemias (data not shown).

To assess further the possible link between *c-myc* expression in variant HCs and their resistance to IFN-α therapy, we studied the *c-myc* mRNA levels during treatment. We observed that even after 6 weeks of IFN-α administration, *c-myc* transcripts persisted at constant levels in variant HCs (Fig 2C), although they remained undetectable in typical HCs at several time points following the first administration of IFN-α (data not shown). In Fig 2B, we also show that the level of *c-fos* mRNA in variant HCs was modified despite the ineffectiveness of IFN-α therapy. In four typical HCL cases in which IFN-α was effective, a sequential study revealed two patterns of *c-fos* expression after the first administration of IFN-α (Fig 2A). In two nonsplenectomized patients, we noticed a rapid disappearance of the *c-fos* transcripts, whereas IFN-α induced a transient accumulation of *c-fos* mRNA in two splenectomized patients. These variations of *c-fos* mRNA levels are not due to a generalized change in the overall level of mRNA as indicated by the nearly constant levels of β-actin mRNA (Figs 2A and 2D).

Thus, the expression of *c-fos*, but not *c-myc*, in variant HCs is modulated by IFN-α administration. The *c-myc* gene is of particular interest because not only is its expression closely associated with cellular proliferation but it also has been shown in vitro that in the case of growth inhibition of cell lines by IFN, a decrease in *c-myc* mRNA accumulation correlates with the antiproliferative activity.[20] Therefore, the failure to modulate *c-myc* expression might be a good indicator of the limits of low-dose IFN-α therapy, even if *c-myc* expression is not solely responsible for the resistant phenotype. The meaning of the variation of *c-fos* expression in response to IFN-α remains unclear.

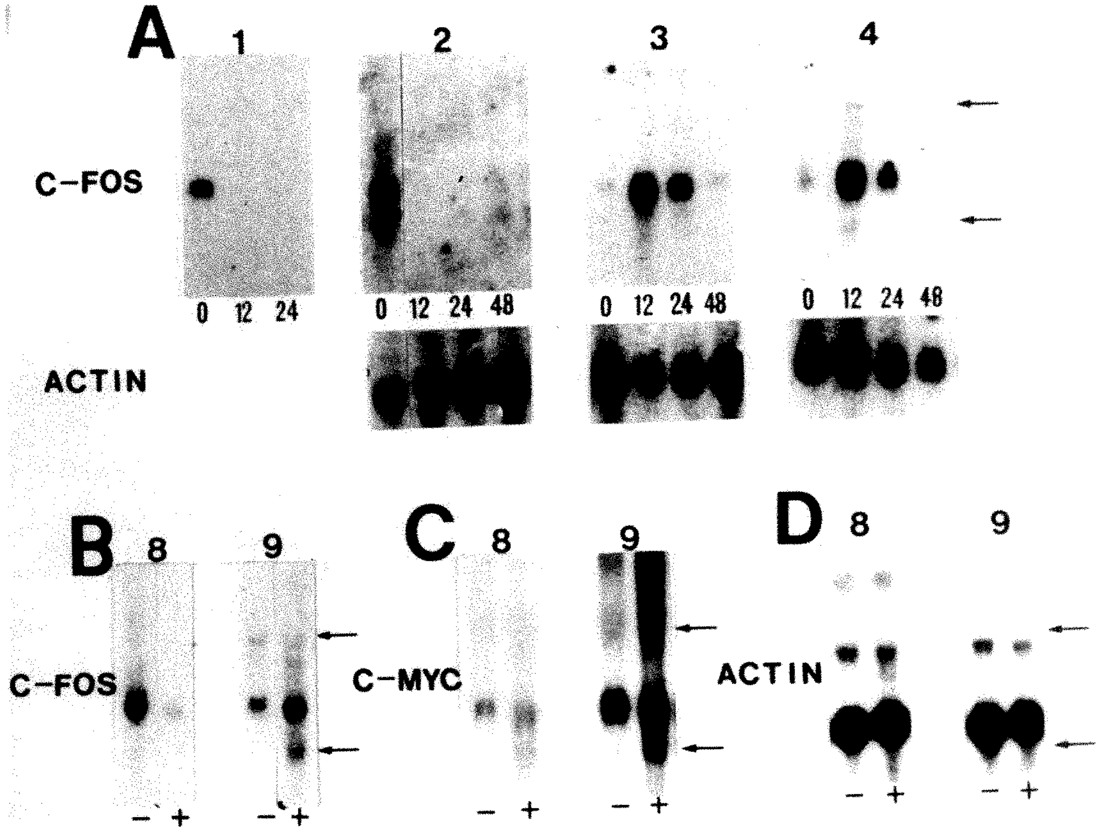

Fig 2. Expression of *c-fos* and *c-myc* during IFN-α therapy. *c-fos* expression (A) is shown in four typical HCL cases (patients 1, 2, 3, and 4 in Table 1) at the indicated hours following the first administration of IFN-α. *c-fos* (B) and *c-myc* (C) expression are depicted in the two variant HCL patients before (−) and after (+) 6 weeks of IFN-α therapy. The arrows indicate the position of the ribosomal RNAs. β-Actin mRNA levels are also shown.

In conclusion, the study of *c-myc* expression will be helpful in the identification of variant HCLs. Our observations suggest that the particular sensitivity of typical HCs to low-dose IFN-α therapy is related to their very low level of proliferation[10] because a specific event of the G0-G1 phase might be the target of IFN action.[9] Thus, although one cannot preclude other mechanisms, we suggest that the cytostatic effect of IFN-α on typical HCs leads to the progressive disappearance of the leukemic cell population by impairing its renewal. Recent data showing that HCs synthesize B cell growth factor (BCGF)[21] and that IFN-α inhibits BCGF-induced proliferation of HCs[22] are consistent with our hypothesis.

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REFERENCES

1. Anderson KC, Boyd AW, Fisher DC, Leslie D, Schlossman SF, Nadler LM: Hairy cell leukemia: A tumor of pre-plasma cells. Blood 65:620, 1985

2. Quesada JR, Reuben JR, Manning JT, Hersh EM, Gutterman JE: Alpha interferon for induction of remission in hairy cell leukemia. N Engl J Med 310:15, 1984

3. Ratain MJ, Golomb HM, Vardiman JW, Vokes EE, Jacobs RH, Daly K: Treatment of hairy cell leukemia with recombinant alpha 2 interferon. Blood 65:644, 1985

4. Jacobs AD, Champlin RE, Golde DW: Recombinant alpha 2 interferon for hairy cell leukemia. Blood 65:1017, 1985

5. Flandrin G, Sigaux F, Castaigne S, Billard C, Aguet M, Boiron M, Falcoff E, Degos L: Treatment of hairy cell leukemia with recombinant alpha interferon: I. Quantitative study of bone marrow changes during the first months of treatment. Blood 67:817, 1986

6. Cawley JC, Burns GF, Hayhoe FGJ: A chronic lymphoproliferative disorder with distinctive features: A distinct variant of hairy cell leukemia. Leuk Res 4:547, 1980

7. Catovsky D, O'Brien M, Melo JV, Wardle J, Brozovic M: Hairy Cell Leukemia (HCL) variant: An intermediate disease between HCL and B prolymphocytic leukemia. Semin Oncol 11:362, 1984

8. Worman CP, Catovsky D, Bevan PC, Camba L, Joyner M, Green PJ, Williams HJH, Bottomley JP, Gordon-Smith EC, Cawley JC: Interferon is effective in hairy cell leukemia. Br J Haematol 60:759, 1985

9. Creasey AA, Bartholomew JC, Merigan TC: Role of G0–G1 arrest in the inhibition of tumor cell growth by interferon. Proc Natl Acad Sci USA 77:1471, 1980

10. Braylan RC, Jaffee ES, Triche TJ, Nanba K, Fowlkes BJ, Metzger H, Frank MM, Dolan MS, Yee CL, Green I, Berard CW: Structural and functional properties of the "hairy" cells of leukemic reticuloendotheliosis. Cancer 41:210, 1978

11. Gonda TJ, Sheiness DK, Bishop JM: Transcripts from the cellular homologs of retroviral oncogenes: distributions among chicken tissues. Mol Cell Biol 2:617, 1982

12. Mitchell KL, Zokas L, Schreiber RD, Verma IM: Rapid induction of the expression of proto-oncogene *fos* during human monocytic differentiation. Cell 40:209, 1985

13. Muller R, Curran T, Muller D, Guilbert L: Induction of *c-fos* during myelomonocytic differentiation and macrophage proliferation. Nature 314:546, 1985

14. Schwarting R, Stein H, Wang CY: The monoclonal antibodies alpha S-HCL1 (alpha leu-14) and alpha S-HCL3 (alpha leu-M5) allow the diagnosis of hairy cell leukemia. Blood 65:974, 1985

15. Maniatis T, Fritsch EF, Sambrook J: Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982

16. Singh L, Jones KW: The use of heparin as a simple cost-effective means of controlling background in nucleic acid hybridization procedures. Nucleic Acids Res 12:5627, 1984

17. Blick M, Westin E, Gutterman J, Wong-Staal F, Gallo R, McCredie K, Keating M, Murphy E: Oncogene expression in human leukemia. Blood 64:1234, 1984

18. Kelly K: The regulation of growth inducible oncogenes in lymphocytes. J Leukocyte Biol 38:47, 1985 (abstr)

19. Grausz JD, Fradelizi D, Dautry F, Monier R, Lehn P: Modulation of *c-fos* and *c-myc* mRNA levels in normal human lymphocytes by calcium ionophore A23187 and phorbol ester. Eur J Immunol (in press)

20. Einat M, Resnitzky D, Kimchi A: Close link between reduction of *c-myc* expression by interferon and G0/G1 arrest. Nature 313:597, 1985

21 Ford RJ, Kwok D, Quesada J, Sahasrabuddhe CG: Production of B cell growth factor(s) by neoplastic B cells from hairy cell leukemia patients. Blood 67:573, 1986

22. Paganelli KA, Evans SS, Han T, Ozer H: B cell growth factor–induced proliferation of hairy cell lymphocytes and inhibition by type I interferon in vitro. Blood 67:937, 1986

CONCISE REPORT

The 18- to 23-kb Deletion of the Macedonian $\delta\beta$-Thalassemia Includes the Entire δ and β Globin Genes

By G.D. Efremov, N. Nikolov, Y. Hattori, I. Bakioglu, and T.H.J. Huisman

Restriction endonuclease mapping analyses were made of DNA from a few members of a Macedonian family with hematological characteristics of $\delta\beta$-thalassemia, ie, microcytosis, normal HbA$_2$ levels, and elevated levels of HbF (7% to 14%) with $^G\gamma$ (average 40.5%) and $^A\gamma^T$ chains (average 59.5%). A large deletion of 18 to 23 kb was present with a 5′ breakpoint within a 670-bp segment of DNA between the *Hpa*I and *Nco*I restriction sites 5′ to the δ globin gene, and a 3′ breakpoint between the *Bam*HI and *Hpa*I restriction sites located some 9 to 13 kb 3′ to the β globin gene. This deletion is different from those present in other types of $G_\gamma A_\gamma(\delta\beta)^\circ$-thalassemia. The similarity of the hematological expression of these $\delta\beta$-thalassemic conditions which have somewhat comparable 5′ breakpoints supports the idea that an important fetal hemoglobin–controlling region lies between the $\psi\beta$ and δ globin genes.

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DESPITE SIMILARITIES in hematological abnormalities in heterozygotes, such as microcytosis, normal HbA$_2$ levels, and elevated HbF levels ranging between 3% and 15%, $\delta\beta$ thalassemia is a mostly heterogeneous condition. All types described thus far are characterized by a relatively large deletion which includes at least part of the δ globin gene and the β globin gene. In some, the deletion is so extensive that it also involves the $^A\gamma$ globin gene,[1-3] while in others both $^G\gamma$ and $^A\gamma$ globin genes are intact.[4,5] In the present paper we provide details about another type of $^G\gamma^A\gamma(\delta\beta)^\circ$-thalassemia which was found in a Macedonian family and is different from the Sicilian[4,6] and Spanish[5,7] types. This Macedonian type of $\delta\beta$-thalassemia has a deletion of 18 to 23 kb with a 5′ endpoint close to the 5′ end of the δ gene, quite similar to that seen in the so-called Sicilian type of $\delta\beta$-thalassemia.[4,5] Phenotypic differences between these two types of $(\delta\beta)^\circ$-thalassemia are negligible.

MATERIALS AND METHODS

Blood samples. These were collected in vacutainers (Becton Dickinson, Rutherford, NJ) with EDTA as anticoagulant, and transported by the first author from Skopje, Yugoslavia to Augusta, Ga. Informed consent was obtained prior to collection.

Hemoglobin analyses. Methodology has been described in earlier publications.[8-10] The previously published $^G\gamma$ to $^A\gamma$ ratios in isolated HbF were confirmed with a more accurate reverse phase high performance liquid chromatographic (HPLC) procedure described by Shelton et al.[11]

DNA analysis. Genomic DNA was isolated from the white cells of 20 mL blood from the propositus (II-4[10]) with the $\delta\beta$-thalassemia-β-thalassemia condition, and from three relatives with a simple $\delta\beta$-thalassemia heterozygosity (father I-5,[10] and two others) using the method described by Poncz et al.[12] Restriction endonuclease digestions were carried out following the directions of the manufacturers (Bethesda Research Laboratories, Bethesda, Md). Fragments were separated on 0.8% agarose gels. Transfer of DNA to nitrocellulose membranes, hybridization to ^{32}P-labelled probes, and autoradiography were made as described in previous publications.[13-16] The four probes used were a cloned *Bam*HI/*Pvu*II fragment containing the γ-IVSII (γ-IVSII probe), a $\gamma\delta$1.6BX probe containing the $\psi\beta$ gene ($\psi\beta$ probe), a 912-bp *Bam*HI/*Eco*RI fragment containing the large IVSII of the β globin gene (β-IVSII probe), and pRK28 containing the 0.8-kb *Bgl*II/*Eco*RI fragment from the region 17 kb 3′ to the β globin gene. The probes were obtained from Dr O. Smithies and collaborators (Madison, Wis), and from Dr R.E. Kaufman (Durham, NC).

RESULTS

The family. This relatively large family from Strumica in Macedonia, Yugoslavia, has been discussed on three occasions in previous communications.[8,9,10] The propositus is presently a 13-year-old girl with a $^G\gamma^A\gamma(\delta\beta)^\circ$-thalassemia-$\beta$-thalassemia condition who inherited the β-thalassemia determinant from the mother and the $\delta\beta$-thalassemia from the father. Nine relatives (including the father) were known to have this $\delta\beta$-thalassemia heterozygosity which is characterized by a distinct microcytosis and mild hypochromia. The HbA$_2$ level averaged 2.5% with a range of 2.1% to 3.1%; HbF by alkali denaturation averaged 10% with a range of 7% to 14%. This HbF contained $^G\gamma$ chains (average 40.5%), $^A\gamma^T$ chains (average 59.5%), and no detectable $^A\gamma^I$ chains. The level of HbF in the propositus was 84%; the $^G\gamma$, $^A\gamma^T$, and $^A\gamma$ values were 58%, 30%, and 12%, respectively, which reflects the dilution effect from the non-$^A\gamma^T$-containing HbF of the β-thalassemia determinant inherited from the mother, who was negative for the $^A\gamma^T$ chain.

DNA analysis. When DNA from one of the $\delta\beta$-thalassemia heterozygotes was digested with a battery of restriction enzymes and analyzed by blot hybridization using a probe specific for the β globin gene (the β-IVSII probe) only normal-sized fragments were observed. However, all bands were more weakly hybridizing than seen for control DNA, indicating the deletion of the β globin gene. Similar analyses with the γ-IVSII probe gave normal-sized fragments; all bands had the same intensities as in the control indicating the

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Table 1. Restriction Endonuclease Data for DNA from a Normal Control and a G$_\gamma$A$_\gamma$($\delta\beta$)$^\circ$-Thalassemia Heterozygote Following Hybridization to the $\psi\beta$ and pRK28 Probes

Enzyme	Control	Patient
Detected with the $\psi\beta$ probe ($\gamma\delta$1.6BX)		
*Bgl*I	11	11
*Eco*RI	7.0	7.0
*Bcl*I	7.4	7.4
*Eco*RV	16; 3.1; 0.9	16; 3.1; 0.9
*Hpa*I	14	14
*Xba*I	7.5	7.5
*Hind*III	18	18*
*Apa*I	21.5	21.5; $\underline{30}$
*Bal*I	16; 5.5	16; $\underline{11}$; 5.5
*Nco*I	8.9	$\underline{10.3}$; 8.9
*Pvu*II	14; 11.5†	$\underline{17}$; 14
*Bam*HI	15.5	15.5; $\underline{16}$
Detected with the pRK28 probe		
*Eco*RI	11	11; $\underline{7.4}$
*Bam*HI	21; 12.7	12.7; $\underline{16}$
*Hpa*I	23	23
*Hind*III	15.3; 13.7	15.3; $\underline{10.5}$‡

Abnormal fragments are underscored.

*Abnormal-sized fragment was not observed.

†Polymorphic site.

‡This 10.5-kb fragment did not hybridize with the $\psi\beta$ probe.

presence of both γ globin genes on the affected chromosome. Hybridization with the $\psi\beta$ probe gave abnormal fragments with five enzymes but not with several others (Table 1 and Fig 1). Evaluation of these data using the restriction map of Fig 2 indicated that the deletion included the *Nco*I restriction site just 5′ to δ, the *Pvu*II and *Bam*HI sites within the δ gene, and the *Hind*III and *Bal*I sites 3′ to δ, but not the *Hpa*I and *Bgl*I sites 5′ to δ. These data indicate that the 5′ breakpoint is located between the *Hpa*I and *Nco*I sites close to the 5′ end of the δ globin gene. This breakpoint is different from that of the Sicilian type of ($\delta\beta$)°-thalassemia; no abnormal *Nco*I, *Bam*HI, and *Pvu*II fragments were present

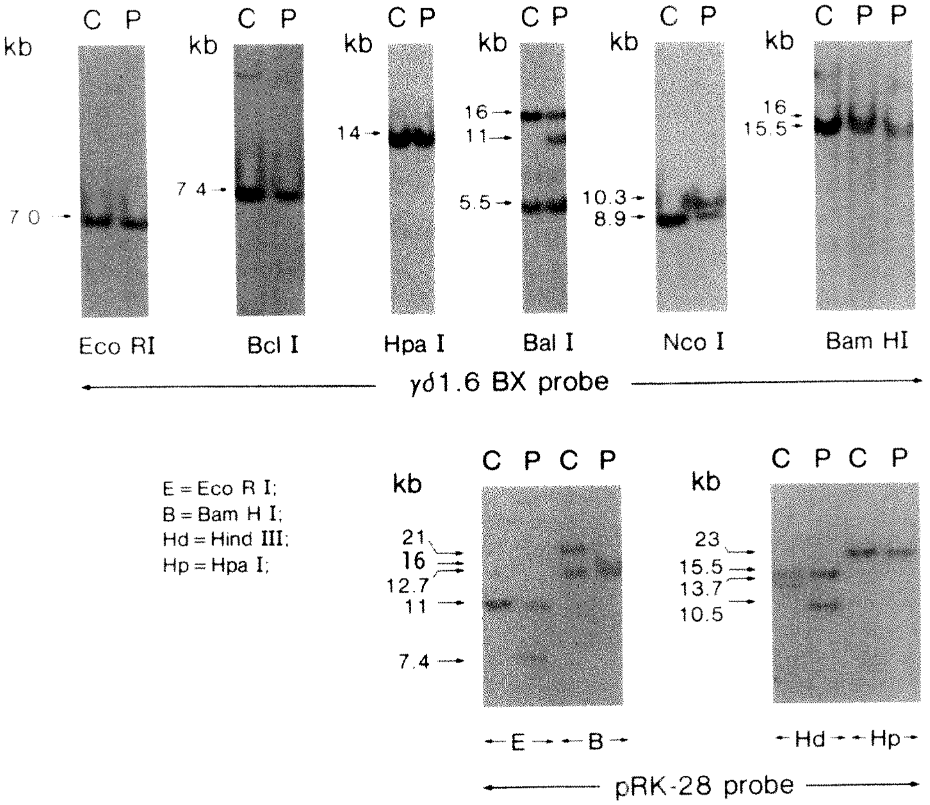

Fig 1. Hybridization of DNA from a normal person (C) and a $^{G}\gamma^{A}\gamma(\delta\beta)^\circ$-thalassemia heterozygote (P) with ^{32}P-labeled probes as indicated.

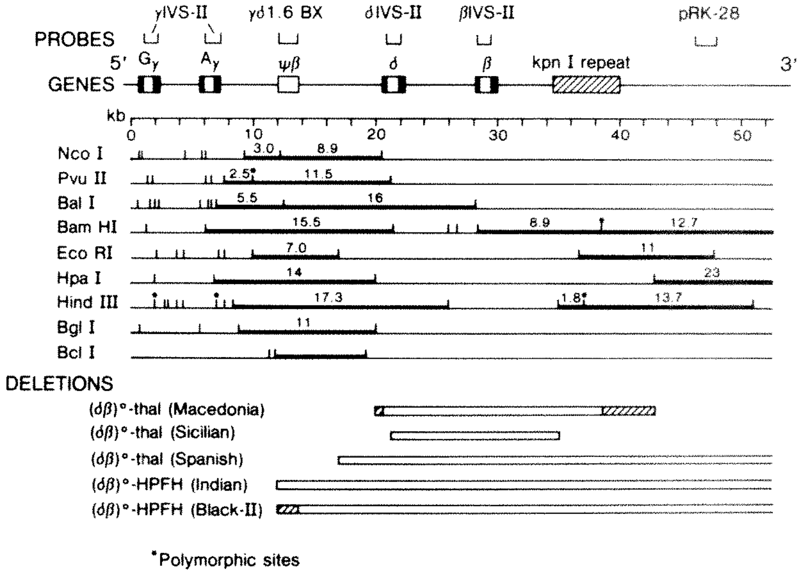

Fig 2. A map listing a limited number of restriction endonuclease cleavage sites in normal human DNA. The fragments identified in the normal control are indicated by the heavy lines and by their size in kb. The location of restriction enzyme sites in normal DNA is from refs 15 and 25. The data indicating the deletions listed in the bottom section are from refs 4, 5, 13, and 24.

in the DNAs of control samples from subjects with a heterozygosity or homozygosity for that condition, while the characteristic large 23-kb *Hind*III fragment was readily observed.[6]

Further experiments involved blot hybridization using the pRK28 probe. A normal-sized *Hpa*I fragment of 23 kb was observed, but abnormal fragments were present in *Eco*RI, *Bam*HI, and *Hind*III digests (Fig 1, Table 1). These results locate the 3' breakpoint of the deletion between the *Bam*HI and *Hpa*I restriction sites (Fig 2). The abnormal *Bam*HI also hybridized with the ψβ probe which made it possible to calculate the extent of the deletion. Thus, the maximum size of the deletion is from a point close to the 5' end of the δ gene to about 13 kb on the 3' side of the β globin gene, or about 23 kb. This conclusion is considered preliminary because of the absence of a large *Hind*III fragment hybridizing with both ψβ and pRK28 probes and the relatively small abnormal Bam HI fragment. Analyses of sequences of a fragment bridging the deletion will be required.

DISCUSSION

In some of the types of (δβ)°-thalassemia that have been characterized in recent years, the deletion involves the β, δ, and ψβ globin genes, as well as one of the γ globin genes ($^{A}\gamma$); as a result the elevated HbF in heterozygotes and homozygotes contains α and $^{G}\gamma$ chains.[1-3] Our Macedonian type of δβ-thalassemia should be compared with the Sicilian and Spanish types; all three have elevated levels of HbF with both $^{G}\gamma$ and $^{A}\gamma$ chains[4-7]. For some time the Macedonian δβ-thalassemia was considered to be similar to the Spanish type; heterozygotes have the same hematological data and morphology, and above all, an elevated HbF with $^{G}\gamma$ and $^{A}\gamma^{T}$ chains in a 40:60 ratio.[9,10] Data from gene mapping analyses, however, indicate that the three (δβ)°-thalassemia types are quite different. The Sicilian δβ-thalassemia has a deletion of about 13 kb, involving part of the δ globin gene and the β globin gene, while that of the Spanish type is much larger with a 3' breakpoint beyond sequences to be recognized by the pRK28 probe (Fig 2). The presence of the two *Hind*III restriction sites, one in each of the two γ globin genes of the chromosome with the Macedonian type of δβ-thalassemia deletion (data not shown), is characteristic for a β globin gene cluster with the $^{A}\gamma^{T}$ globin gene (ie, the mutation in the $^{A}\gamma$ globin gene resulting in the γ^{75} Ile → Thr substitution[17]) 3' to the $^{G}\gamma$ gene, and has also been observed for the chromosome with the Spanish (δβ)°-thalassemia determinant.[7] The deletion in the Macedonian δβ-thalassemia is also different from that observed in a Japanese δβ-thalassemia homozygote[18]; the Japanese deletion is larger with a 5' end located in the vicinity of the 3' end of the $^{A}\gamma$ globin gene.

A comparison of the deletions of the Sicilian, Spanish, and Macedonian (δβ)°-thalassemias with the Indian type of (δβ)°-HPFH (hereditary persistence of fetal hemoglobin) (type III[13]) and with a Black type of (δβ)°-HPFH (Ghanaian type,[19] or type II[13]) shows that the 5' ends of the deletions are quite different (Fig 2). The 3' ends of the deletions in type III[20] and type II[21] HPFH are far beyond the sequences recognized by the pRK28 probe, and thus vastly different from the 3' ends of the Sicilian and Macedonian (δβ)°-thalassemia types but not necessarily from that of the Spanish type. In all three types of δβ-thalassemia a region between the ψβ and δ globin genes, which has been presumed important for controlling fetal hemoglobin production,[22,23] is not part of the deletion, as is the case for the HPFH abnormalities with their distinctly different phenotypic expression. Thus, one wonders if deletion of that region is indeed not responsible for the greatly increased level of HbF in the HPFH heterozygote, while the unknown sequences, newly introduced 5' to or within the δ globin gene, may have a much less significantly enhancing effect on the production of both $^{G}\gamma$ and $^{A}\gamma$ chains than has been suggested.[24,25] However, the larger deletion in the Japanese δβ-thalassemia condition includes the same region, albeit resulting in a different hematological condition. These data do not fit this explanation, suggesting that other mechanisms may also effect the expression of the γ globin genes in these $\delta\beta$-thalassemia conditions.[18]

REFERENCES

1. Trent RJ, Jones RW, Clegg JB, Weatherall DJ, Davidson R, Wood WG: $(^{A}\gamma\delta\beta)^{\circ}$-thalassaemia: Similarity of phenotype in four different molecular defects, including one newly described. Br J Haematol 57:279, 1984

2. Henthorn PS, Smithies O, Nakatsuji T, Felice AE, Gardiner MB, Reese AL, Huisman THJ: $(^{A}\gamma\delta\beta)^{\circ}$-thalassaemia in blacks is due to a deletion of 34 kbp of DNA. Br J Haematol 59:343, 1985

3. Zeng Y-T, Huang S-Z, Chen B, Liang Y-C, Liang Z-M, Harano T, Huisman THJ: Hereditary persistence of fetal hemoglobin or $(\delta\beta)^{\circ}$-thalassemia: Three types observed in south Chinese families. Blood 66:1430, 1985

4. Ottolenghi S, Giglioni B: The deletion in a type of $\delta^{\circ}\beta^{\circ}$-thalassaemia begins in an inverted Alu I repeat. Nature 300:770, 1982

5. Ottolenghi S, Giglioni B, Taramelli R, Comi P, Mazza U, Saglio G, Camaschella C, Izzo P, Cao A, Galanello R, Gimferrer E, Baiget M, Gianni AM: Molecular comparison of $\delta\beta$-thalassemia and hereditary persistence of fetal hemoglobin DNAs: Evidence of a regulatory area? Proc Natl Acad Sci USA 79:2347, 1982

6. Efremov GD, Filipce V, Gjorgovski I, Juricic D, Stojanovski N, Harano T, Nakatsuji T, Kutlar A, Kutlar F, Bakioglu I, Huisman THJ: $^{G}\gamma^{A}\gamma$ $(\delta\beta)^{\circ}$-thalassemia and a new form of γ globin gene triplication identified in the Yugoslavian population. Br J Haematol 63:17, 1986

7. Huisman THJ, Kutlar F, Nakatsuji T, Bruce-Tagoe A, Kilinc Y, Cauchi MN, Romero Garcia C: The frequency of the γ chain variant $^{A}\gamma^{T}$ in different populations, and its use in evaluating γ gene expression in association with thalassemia. Hum Genet 71:127, 1985

8. Efremov GD, Nikolov N, Duma H, Schroeder WA, Miller A, Huisman THJ: $\delta\beta$-Thalassaemia in two Yugoslavian families. Scand J Haematol 14:226, 1975

9. Schroeder WA, Huisman THJ, Efremov GD, Shelton JB, Shelton JR, Phillips R, Reese A, Gravely M, Harrison JM, Lam H: Further studies of the frequency and significance of the $^{T}\gamma$-chain of human fetal hemoglobin. J Clin Invest 63:268, 1979

10. Efremov GD, Ibarra B, Gurgey A, Sukumaran PK, Altay C, Huisman THJ: Gamma-chain heterogeneity of fetal hemoglobin in non-black β- and $\delta\beta$-thalassemia and HPFH heterozygotes and homozygotes. Am J Hematol 12:367, 1982

11. Shelton JB, Shelton JR, Schroeder WA: High performance liquid chromatographic separation of globin chains on a large-pore C_4 column. J Chromatogr 7:1969, 1984

12. Poncz M, Solowiejczyk D, Harpel B, Mory Y, Schwartz E, Surrey S: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β-like globin genes. Hemoglobin 6:27, 1982

13. Kutlar A, Gardiner MB, Headlee MG, Reese AL, Cleek MP, Nagle S, Sukumaran PK, Huisman THJ: Heterogeneity in the molecular basis of three types of hereditary persistence of fetal hemoglobin and the relative synthesis of the $^{G}\gamma$ and $^{A}\gamma$ types of γ chain. Biochem Genet 22:21, 1984

14. Sukumaran PK, Nakatsuji T, Gardiner MB, Reese AL, Gilman JG, Huisman THJ: Gamma thalassemia resulting from the deletion of a γ-globin gene. Nucleic Acids Res 11:4635, 1983.

15. Gilman JG, Huisman THJ, Abels J: Dutch β°-thalassaemia: A 10 kilobase DNA deletion associated with significant γ-chain production. Br J Haematol 56:339, 1984.

16. Padanilam BJ, Felice AE, Huisman THJ: Partial deletion of the 5′ β-globin gene region causes β°-thalassemia in members of an American black family. Blood 64:941, 1984

17. Grifoni V, Kamuzora H, Lehmann H, Charlesworth D: A new Hb variant: Hb F Sardinia γ75(E 19) Isoleucine → Threonine found in a family with Hb G Philadelphia, β-chain deficiency and Lepore-like haemoglobin indistinguishable from Hb A_2. Acta Haematol 53:347, 1975

18. Matsunaga E, Kimura A, Yamada H, Fukumaki Y, Takagi Y: A novel deletion in $\delta\beta$-thalassemia found in Japan. Biochem Biophys Res Commun 126:185, 1985

19. Tuan D, Murnane MJ, deRiel JK, Forget BG: Heterogeneity in the molecular basis of hereditary persistence of fetal haemoglobin. Nature 285:335, 1980

20. Henthorn PS: Three deletions in the human β-globin gene cluster: sequence analysis and length relationship. Doctoral dissertation, University of Wisconsin, Madison, 1984

21. Vanin EF, Henthorn PS, Koussis D, Grosveld F, Smithies O: Unexpected relationships between four large deletions in the human β-globin gene cluster. Cell 35:701, 1983

22. Huisman THJ, Schroeder WA, Efremov GD, Duma H, Mladenovski B, Hyman CB, Rachmilewitz EA, Bouver N, Miller A, Brodie A, Shelton JR, Shelton JB, Apell G: The present status of the heterogeneity of fetal hemoglobin in β-thalassemia: An attempt to unify some observations in thalassemia and related conditions. Ann NY Acad Sci 232:107, 1974

23. Weatherall DJ, Clegg JB: The Thalassaemia Syndromes (ed 3). Blackwell, Oxford, 1981

24. Tuan D, Feingold E, Newman M, Weissman SM, Forget BG: Different endpoints of deletions causing $\delta\beta$-thalassemia and hereditary persistence of fetal hemoglobin: Implications for the control of γ-globin gene expression in man. Proc Natl Acad Sci USA 80:6937, 1983

25. Fritsch EF, Lawn RM, Maniatis T: Characterization of deletions which affect the expression of fetal globin genes in man. Nature 279:598, 1983

CONCISE REPORT

Acute Lymphoblastic Leukemia in Infants: Evidence for B Cell Origin of Disease by Use of Monoclonal Antibody Phenotyping

By Patricia A. Dinndorf and Gregory H. Reaman

Since the prognosis of infants with acute lymphoblastic leukemia (ALL) is so poor, it has been suggested that these leukemias may not be lymphoid in origin, but may originate from stem cell, myeloid, or megakaryocytic progenitors. Alternately it has been hypothesized that these leukemias originate in lymphoid cells at the earliest stages of B cell development. Another possibility is that these leukemias may be of more than one lineage. Therefore we examined leukemic blasts from 12 infants with ALL using monoclonal antibodies to myeloid and lymphoid differentiation antigens. The majority of specimens expressed HLA/DR and reacted with B4 (CD19) but failed to react with stem cell, myeloid, megakaryocytic, or T cell associated antibodies. These results support the speculation that the majority of these leukemias arise in cells at the earliest stages of B cell commitment, and are not of a myeloid or biphenotypic nature.

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THE MAJORITY of patients diagnosed with acute lymphoblastic leukemia (ALL) in childhood can expect to experience long-term disease-free survival.[1] Infants less than one year of age at diagnosis experience a distinctively worse outcome.[2-7] Prognostic factors which have been identified in ALL include initial white count, age, sex, presence of mass disease, and importantly, the immunologic phenotype of the leukemic cells.[1,8] The majority of patients can be classified into groups whose leukemic cells originate from lymphoid cells committed to T cell or B cell differentiation as defined by their cell surface characteristics or genomic rearrangements.[9-11] Thus leukemic cells presumably originate from normal cellular counterparts exhibiting well-defined features of discrete stages of differentiation, which define biologically important characteristics of the disease.

The majority of infants with ALL exhibit many of the clinical factors associated with a poor prognosis, including hyperleukocytosis, hepatosplenomegaly, and meningeal leukemia at presentation.[2,3] It has been noted that leukemic cells from these patients generally fail to express antigens associated with T cell (E-rosette receptor), B cell (surface immunoglobulin), or B cell commitment (ie, the common acute lymphocytic leukemia antigen [CALLA]) but express only HLA/DR.[3] Because infants with ALL have such a poor prognosis, it has been suggested that the leukemic cell in this disease may not necessarily be lymphoid in origin, but may originate from pluripotent stem cells or megakaryocytes, or may in fact be of myeloid origin. Alternately, it has been suggested that these leukemias may originate from early lymphoid cells committed to B cell differentiation that have not yet acquired CALLA, but express only the HLA/DR antigen.[3,12] It is also possible that they may exhibit antigenic determinants of more than one lineage, a characteristic which has been recently reported to predict a poor outcome for ALL in adults; the expression of myeloid antigens by leukemic blasts of adult patients with ALL was recently demonstrated to define a group of patients with a distinctively worse prognosis.[13]

In order to determine the cell of origin of these leukemias, we used a panel of monoclonal antibodies including one that reacts with stem cells, as well as antibodies to myeloid and megakaryocytic determinants, to examine blasts from 12 infants with newly diagnosed ALL. In addition, antibodies that react with lymphoid cells were used to examine the differentiative state of these leukemic cells.

MATERIALS AND METHODS

Cells. Samples of heparinized marrow were collected at the time of initial diagnosis of leukemia. Parental informed consent was obtained. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (specific gravity 1.077), and were cryopreserved in a viable state with 10% dimethylsulfoxide and maintained in the vapor phase of liquid nitrogen. Prior to immunofluorescent assays, cells were rapidly thawed and washed three times. All samples had at least 85% malignant blasts as ascertained by examination of Wright-stained cytospin preparations. Viability was assessed by trypan blue exclusion, and was greater than 75% in all cases.

Antibodies. Monoclonal antibodies were used in the form of ascites fluid at dilutions of 1:250 to 1:500. The antibodies and their specificities are presented in Table 1. 12-8, 1G10, 5F1, and L4F3 were provided by Dr Irwin D. Bernstein, antibodies C7E10 and 13.1 were donated by Dr Diane Nugent, and antibodies 4.1 and 9.6 were obtained from Dr Paul Martin, Fred Hutchinson Cancer Research Center, Seattle, Wash. Antibody B4 was a gift from Dr Kenneth Anderson, Dana Farber Institute, Boston, Mass.

Immunofluorescent studies. Cells were incubated for 30 minutes with ascites fluid containing monoclonal antibodies, washed with phosphate-buffered saline (PBS) and then incubated for 30 minutes with affinity-purified, fluorescein-conjugated isotype-specific goat antimouse immunoglobulin at a dilution of 1:100 (Tago, Burlingame, Calif). Cells were examined by fluorescent microscopy and 200 cells were counted for each assay. Samples with greater than 15% cells demonstrating reactivity with the antibody were considered positive.

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Blood, Vol 68, No 4 (October), 1986: pp 975-978

Table 1. Specificities of Antibodies and Results of Immunofluorescent Studies on Leukemia Cells

Antibody	Antigen	Major Reactivity	Results*
Stem Cell			
12-8[14]	115 Kd	CFU-GM, BFU-E, CFU-Mix	1/12
Myeloid			
1G10(CD15)[15,16]	X-hapten	Granulocytes	0/11
L4F3[15,16]	62 Kd	Immature myeloid cells	0/9
5F1(CDw14)[15,16]	85 Kd	Monocytes, nucleated red cells, platelets	0/11
Megakaryocytic			
C7E10[17]	glycoprotein Ib	Megakaryocytes, platelets	0/12
13.1	glycoprotein IIb/IIIa	Megakaryocytes, platelets	0/12
B Cell–Associated			
4.1[18]	HLA/DR		9/11
B4(CD19)[19]	95 Kd	Pan-B cell	11/12
BA-3(CD10)[20]	100 Kd	CALLA	2/12
T cell			
9.6/OKT11(CD2)[21]	E-rosette	Pan-T cell	0/12

*Results are the number of patients whose leukemic cell reacted with the antibody per number of patients tested.

RESULTS

Clinical characteristics of the patients. The patients included in this study ranged from 1 month to 11 months of age at diagnosis. The mean white count was $2.8 \times 10^5/\mu L$ (range 8.7×10^3 to $9.9 \times 10^5/\mu L$). Only one patient had a significant proportion (greater than 25%) of cells which exhibited L2 FAB (French-American-British) morphology. Three patients had central nervous system (CNS) disease at diagnosis.

Reactivity of antibodies with leukemic specimens. The reactivity of these samples with the monoclonal antibodies is shown in Table 1. All samples tested failed to react with myeloid antibodies 1G10, 5F1, or L4F3, and also failed to react with the antimegakaryocytic antibodies 13.1 and C7E10. One patient (the first patient presented on Table 2) reacted with 12-8. None of the blasts tested expressed the E-rosette receptor, or expressed surface immunoglobulin.

Reactivity of the individual samples with antibodies to the B cell–associated antigens is presented in Table 2; the patients are presented in chronological order of age at diagnosis. The majority of samples reacted with 4.1, the anti-HLA/DR antibody, and B4. This pattern is seen on normal lymphocytes at the earliest stages of commitment to B cell differentiation. Two samples also expressed CALLA, an antigen that is expressed later in B cell differentiation. Of note, these patients were older than 6 months of age at diagnosis. In addition, another one of the older infants expressed CALLA on 15% of her cells.

DISCUSSION

Infants with ALL have the worst prognosis of any group of children with this disease.[2-7] Infants with ALL exhibit many of the poor prognostic clinical characteristics that have been identified, but the outcome of these children is still worse than that predicted for older patients (greater than 1 year of age) with the same adverse features.[2,3] These leukemias generally fail to exhibit T cell characteristics or express CALLA, although the majority express HLA/DR.[3] The absence of specific lymphoid lineage–associated makers, has led to speculation that these leukemias may not be lymphoid in origin. It has been suggested that they may originate from a pluripotent stem cell, micromegakaryocytes, or myeloid cells. This has important implications for therapy; the strategy of ablative induction therapy used in the treatment of acute nonlymphocytic leukemia may be a better approach. A recent report suggested that patients with ALL whose blast cells expressed myeloid-associated antigens experienced a worse response to therapy than those whose cells expressed exclusively lymphoid antigens.[13] Thus, expression of myeloid-associated antigens on the blasts of infants with

Table 2. B Cell–Associated Antigen Expression of Leukemic Cells

Pre-B Stage I ——→
Stage II ————→
Stage III ——————→

Age (mos)	HLA/DR	B4	CALLA
11	92	96	—
11	92	95	—^{15}
10	ND	—	77
10	—	32	36
8	95	83	—
7	96	95	—
6	69	78	—
5	95	85	—
4	97	89	—
3	—	92	—
2	94	93	—
1	92	93	—

The results are reported as the percent of cells expressing the antigen for the positive samples and specimens with less than 15% are reported as —. ND, not done.

ALL as an explanation for this dismal response to therapy required investigation.

Others have suggested, however, that these leukemias may originate from cells in the earliest stages of commitment to B cell differentiation.[3,12] Nadler et al have presented a schema of antigen expression during B cell differentiation.[12] According to this schema, the first antigen to be expressed is HLA/DR, corresponding to stage I (Table 2). At the level of stage II development the surface determinant identified by the monoclonal antibody B4 is expressed. At the genomic level this is the stage where heavy chain immunoglobulin gene rearrangements occur. Lymphoblasts at stage III express CALLA and begin to demonstrate immunoglobulin light chain gene rearrangements. Subsequently, these cells are at the pre-B level when they develop cytoplasmic immunoglobulin. Finally, surface immunoglobulin is expressed and the cells are mature B cells. Results of phenotyping by these investigators demonstrated that a higher proportion of ALL in children less than 2 years of age exhibited the stage II pattern. In this study the number of patients less than 1 year of age (infants) is not reported, so it is unclear what portion of these patients were infants.

The present study of infants less than 1 year of age with ALL confirms the hypothesis that the leukemic cells in this disease originate from a lymphoid cell at an early stage of commitment to B cell differentiation (stage II). Of note, it was the infants greater than 6 months of age in this series whose leukemic cells expressed CALLA (stage III).

The leukemic cells of 11 of the 12 infants tested failed to react with antibody 12-8, which is expressed on nearly all unipotent and multipotent colony-forming cells. This suggests that these leukemias do not originate from undifferentiated stem cells. The leukemic cells of all 12 infants also failed to react with monoclonal antibodies 1G10 and 5F1, antibodies that detect myeloid differentiation antigens. The nine samples incubated with L4F3 also failed to react with this myeloid differentiation antibody. L4F3 reacts with the same antigen identified by MY-9.[14] MY-9 was one of the two antibodies that were useful in the CALGB study in identifying leukemias with mixed lymphoid and myeloid characteristics that displayed a worse response to therapy.[13] In previous studies we have demonstrated that in 95% of cases of childhood acute nonlymphocytic leukemia the leukemic blasts react with one of these antibodies.[16,22]

Finally, it has been suggested that ALL in infants may in fact represent a micromegakaryocytic variety of acute nonlymphocytic leukemia. None of the cases tested reacted with C7E10, the antiglycoprotein Ib, or 13.1, the antiglycoprotein IIb/IIIa. It has been demonstrated that megakaryocytic leukemias express these glycoprotein antigens.[23]

These data suggest that ALL in infants originates in lymphoid cells at a very early stage of B cell differentiation and not from other hematopoietic progenitors, nor do their leukemic cells express antigens of mixed myeloid and lymphoid origin. Phenotypic analysis of the leukemic blast cells of a larger number of these patients may provide some biologic insights to explain their poor outcome. It will also be of interest to analyze whether this early B cell phenotype defines a group of older patients who experience a worse response to therapy.

REFERENCES

1. Bleyer WA: Acute lymphoid leukemia. Pediatr Ann 12:277, 1983

2. Reaman G, Zeltzer P, Bleyer WA, Amendola B, Level C, Sather H, Hammond D: Acute lymphoblastic leukemia in infants less than one year of age: A cumulative experience of the Children's Cancer Study Group. J Clin Oncol 3:1513, 1985

3. Crist W, Pullen J, Boyett J, Falletta J, van Eys J, Borowitz M, Jackson J, Dowell B, Frankel L, Quddus F, Ragab A, Vietti T: Clinical and biologic features predict a poor prognosis in acute lymphoid leukemias in infants: A Pediatric Oncology Group Study. Blood 67:135, 1986

4. Cangir A, George S, Sullivan M: Unfavorable prognosis of acute leukemia in infancy. Cancer 36:173, 1975

5. Kardos G, Revesz T, Koos R, Petroczky A, Schuler D: Infant leukemia in Hungary. Med Pediatr Oncol 10:201, 1982

6. Finkelstein JZ, Higgins GR, Rissman E, Nixon GW: Acute leukemia during the first year of life. Presentation, chemotherapy and clinical course. Clin Pediatr 11:236, 1972

7. Pierce MI: Leukemia in the newborn infant. J Pediatr 54:691, 1959

8. Robison LL, Sather HN, Coccia PF, Nesbit ME, Hammond GD: Assessment of the interrelationship of prognostic factors in childhood acute lymphoblastic leukemia. Am J Pediatr Hematol Oncol 2:5, 1980

9. Foon KA, Schroff RW, Gale RD: Surface markers on leukemia and lymphoma cells: Recent advances. Blood 60:1, 1982

10. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA: Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemia of T cell and B cell precursor origins. J Clin Invest 71:301, 1983

11. Waldman TA, Davis MM, Bongiovanni KF, Korsmeyer SJ: Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 313:776, 1985

12. Nadler LM, Korsmeyer SJ, Anderson KC, Boyd AW, Slaughenhoupt B, Park E, Jensen J, Coral F, Mayer RJ, Sallon SE, Ritz J, Schlossman SF: B cell origin of non–T cell acute lymphoblastic leukemia: A model for discrete stages of neoplastic and normal pre–B cell differentiation. J Clin Invest 74:332, 1984

13. Sobol RE, Royston I, Mick R, Cuttner J, Ellison RR, Davey F, Nelson D, Bloomfield CD: Clinical significance of mixed lymphoid and myeloid characteristics in adult acute lymphoblastic leukemia. Proc Am Soc Clin Oncol 5:623, 1986 (abstr)

14. Andrews RG, Singer JW, Bernstein ID: Monoclonal antibody 12-8 recognizes a 115 kd molecule present on both unipotent and multipotent hematopoetic colony-forming cells and their precursors. Blood 67:842, 1986

15. Andrews RG, Torok-Storb B, Bernstein ID: Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood 62:124, 1983

16. Dinndorf PA, Andrews GR, Benjamin D, Ridgway D, Wolfe L, Bernstein ID: Expression of normal myeloid associated antigens by acute leukemia cells. Blood 67:1048, 1986

17. Nugent D, Kunicki T, Montgomery R, Bernstein ID: Monoclonal antiplatelet antibody specific for GP Ib which blocks ristocetin induced agglutination but does not bind to glycocalycin on

crossed immunoelectrophoresis. Thromb Res (submitted for publication)

18. Nepom BS, Nepom GT, Mickelson E, Antonelli P, Hansen JA: Electrophoretic analysis of human HLA-DR antigens from HLA-DR4 homozygous cell lines: Correlation between B chain diversity and HLA-D. Proc Natl Acad Sci USA 80:6962, 1983

19. Anderson KC, Bates MP, Slaughenhoupt BL, Pinkus GS, Schlossman SF, Nadler LM: Expression of human B cell–associated antigens on leukemias and lymphomas: A model of human B cell differentiation. Blood 63:1424, 1984

20. LeBien TW, Boue DR, Bradley JG, Kersey JH: A monoclonal antibody to the common ALL antigen which does not mediate antigenic modulation in vitro. J Cell Biochem 6:34, 1982 (suppl)

21. Hansen JA, Martin PJ: Hybridomas in cancer diagnosis and treatment, in Mitchell MS, Oettgen HF (eds): Progress in Cancer Research and Therapy Series (vol 21). New York, Raven, 1982, p 75

22. Dinndorf PA, Benjamin D, Ridgway D, Bernstein ID: Immunodiagnosis of childhood ALL with antibodies to myeloid and lymphoid associated antigens. Leuk Res 9:449, 1985

23. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7): A report of the French-American-British Cooperative Group. Ann Intern Med 103:460, 1985

CORRESPONDENCE

PHARMACOKINETICS OF FACTOR IX

To the Editor:

The recent article by Noe et al[1] showed that the steady state level of factor IX and factor VIII in patients treated with constant infusions allowed calculation of the metabolic clearance of factors IX and VIII. The authors noted that the dose of factor IX given was linearly related to the factor IX level but did not note whether there was any effect of endogenous factor IX antigen levels or factor IX dose on the metabolic clearance rate determined in eight factor IX deficient patients.

Bovine factor IX binds to cultured endothelial cells and on bovine aortic slices,[2,3] and Fuchs et al have shown that human factor IX clearance in mice is affected by the dose of factor IX given.[4] These observations suggest that factor IX kinetics in humans should be nonlinear, since the rate of disappearance probably is affected by the amount of factor IX bound to endothelial cells. Data in prior studies, however, seem to have been adequately described by the linear two-compartment pharmacokinetic model.[5,6]

The data provided by Noe et al were examined to see if factor IX follows a linear pharmacokinetic model,[7] and it appears that the metabolic clearance of factor IX expressed in mL/kg/hour is linearly related to dose in U/kg/hour. This relationship holds even if data for repeated infusions in individual patients are averaged to eliminate the potential bias of repeated studies in one patient on the overall data. This relationship of dose and clearance did not hold for the factor VIII data ($r = 0.26$). Table 1 below and Fig 1 illustrate the relationship when the clearance and dose data provided by Noe et al are normalized for weight in kilograms.

Table 1. Factor IX Dose and Clearance in Eight Hemophilia B Patients

Patient	Infusion*	Dose (U/kg/hr)	Clearance (mL/kg/hr)
1	a	1.36	3.77
	b	1.56	4.00
2		3.04	5.83
3		2.37	3.00
4	a	0.78	2.23
	b	1.71	5.04
5	a	1.09	2.42
	b	1.38	3.36
	c	1.10	2.20
	d	1.04	3.85
	e	0.51	2.33
	f	0.26	2.56
6	a	1.02	3.04
	b	0.27	3.01
7	a	1.59	3.11
	b	1.32	4.39
	c	0.66	2.75
8		0.61	1.89

*Repeated infusion studies for patients 1, 4, 5, 6, and 7 are designated a-f.

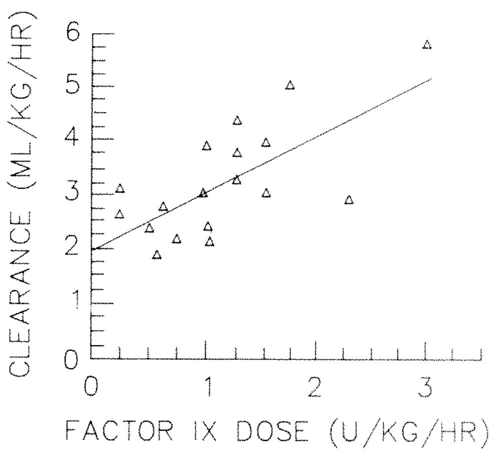

Fig 1. Factor IX clearance at indicated doses for 18 infusions in eight patients reported by Noe et al.[1] The correlation coefficient for the least squares regression line shown is 0.71.

The coefficient of determination is 0.50 and the correlation coefficient is 0.71. The correlation is significant at the $P < 0.001$ level.[8] If factor IX infusion followed first-order pharmacokinetics, clearance should be the product of the volume of distribution of factor IX and its late phase or beta half-life, neither of which should change with dose of factor IX given.[7]

These data support the hypothesis that the endothelial cell receptor for factor IX has an important effect on factor IX clearance, perhaps by reversibly binding a portion of the infused material. The catabolism of factor IX by human vascular endothelial cells cannot be excluded, although this has not been noted in studies of bovine factor IX endothelial cell binding. It would be helpful if the authors could provide data on the factor IX antigen levels of these patients prior to transfusion, since this could also affect the endothelial cell binding site. The response to factor IX may not be dramatically different at high *versus* low doses of factor IX, but in individual patients with low endogenous antigen levels less of an increase in plasma factor IX for a given dose could be expected if the endothelial cell site is not occupied

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REFERENCES

1. Noe DA, Bell WR, Ness PM, Levin J: Plasma clearance rates of coagulation factors VIII and IX in factor-deficient individuals. Blood 67:969, 1986

2. Stern DM, Drillings M, Nossel HL, Hurlet-Jensen A, LaGamma KS, Owen J: Binding of factors IX and IX$_a$ to cultured vascular endothelial cells. Proc Natl Acad Sci USA 80:4119, 1983

3. Stern D, Nawroth P, Kisiel W, Handley D, Drillings M, Bartos J: A coagulation pathway on bovine aortic segments leading to generating factor X$_a$ and thrombin. J Clin Invest 74:1910, 1984

4. Fuchs HE, Trapp HG, Griffith MJ, Roberts HR, Pizzo SV: Regulation of factor IX$_a$ in vitro in human and mouse plasma and in vivo in the mouse. J Clin Invest 73:1696, 1984

5. Zauber NP, Levin J: Factor IX levels in patients with hemophilia B (Christmas disease) following transfusion with concentrates of factor IX or fresh frozen plasma (FFP). Medicine 56:213, 1977

6. Smith KJ, Thompson AR: Labeled factor IX kinetics in patients with hemophilia-B. Blood 58:625, 1981

7. Greenblat DJ, Kock-Wesser J: Clinical Pharmacokinetics (first of two parts). N Engl J Med 293:702, 1975

8. Zar JH: Biostatistical analysis. Englewood Cliffs, NJ, Prentice-Hall, 1974, p 207

To the Editor:

An important question to address in any pharmacokinetic study is whether the system behaves in a linear or a nonlinear fashion. In his letter, Dr Smith argues that factor IX kinetics should not be linear because of the potential for nonlinearity in its elimination and/or nonlinearity in its distribution due to tissue receptor binding. Our data indicate that throughout the dosing range we studied, factor IX administration by continuous infusion behaves linearly. The evidence for linearity is the excellent fit of the proportional model, ie, the regression line passing through the origin, to the relationship between the factor IX infusion rates and the plasma factor IX activities achieved, as shown in our Fig 1.[1] This finding demonstrates that the Superposition Principle is obeyed by the system. The Superposition Principle states that the response of a system to a linear combination of inputs is equal to the sum of the responses of the system to the individual inputs. This principle is "... a unique property of linear systems; it does not hold for nonlinear systems."[2]

A mathematically equivalent way of demonstrating the Superposition Principle is by showing that the average factor IX clearance rate is constant over the dosing range. This is the approach taken by Dr Smith. He finds that this test of linearity fails when both the clearance rates and the infusion rates are normalized for body weight. The statistical significance of this correlation is spurious, however, because the value of the correlation is inflated by having both of the variates expressed as ratios generated by division by the same third variate,[3] which in this case is body weight. The correlation of the unnormalized data does not achieve significance (correlation coefficient 0.43; $0.05 < P < 0.1$).

It should also be noted that neither reversible binding of factor IX to endothelial cells nor elimination by a saturable process such as irreversible receptor-mediated uptake by endothelial cells would result in the clearance rate of factor IX increasing with increasing infusion rates as in Dr Smith's Fig 1. Tissue binding increases the volume of distribution of a substance but does not affect its clearance rate in the steady state.[4] Saturable elimination processes show decreased clearance rates at increased infusion rates.[5]

Finally, we must correct the erroneous definition of clearance rate given by Dr Smith. Greenblatt and Koch-Weser state that, for a bolus dose in a two-compartment linear pharmacokinetic system, clearance rate is equal to the so-called "area" volume of distribution times 0.693 divided by the beta phase half-life.[6] An expression for clearance rate which is much more useful as it applies to systems with any number of kinetic compartments and for any dosing scheme, is that clearance rate equals the administered dose divided by the area under the concentration-time curve.[7]

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REFERENCES

1. Noe DA, Bell WR, Ness PM, Levin J: Plasma clearance rates of coagulation factors VIII and IX in factor-deficient individuals. Blood 67:969, 1986

2. Jacquez JA: Compartmental Analysis in Biology and Medicine. New York, Elsevier, 1972, p 18

3. Pearson K: On a form of spurious correlation which may arise when indices are used in the measurement of organs. Proc R Soc Lond 60:489, 1897

4. Gibaldi M, Perrier D: Pharmacokinetics (ed 2). New York, Dekker, 1982, p 307

5. Gibaldi M, Perrier D: Pharmacokinetics (ed 2). New York, Dekker, 1982, p 287

6. Greenblatt DJ, Koch-Weser J: Clinical pharmacokinetics (first of two parts). N Engl J Med 293:702, 1975

7. DiStefano JJ: Concepts, properties, measurement, and computation of clearance rates of hormones and other substances in biological systems. Ann Biomed Eng 4:302, 1976

ERRATA

In the article entitled "Ultrastructural Localization of Coagulation Factor V in Human Platelets" by Wencel-Drake et al which appeared in the July 1986 issue (Volume 68, Number 1, pages 244–249), Figure 2 was not clearly reproduced in some copies of the journal. It is reprinted below.

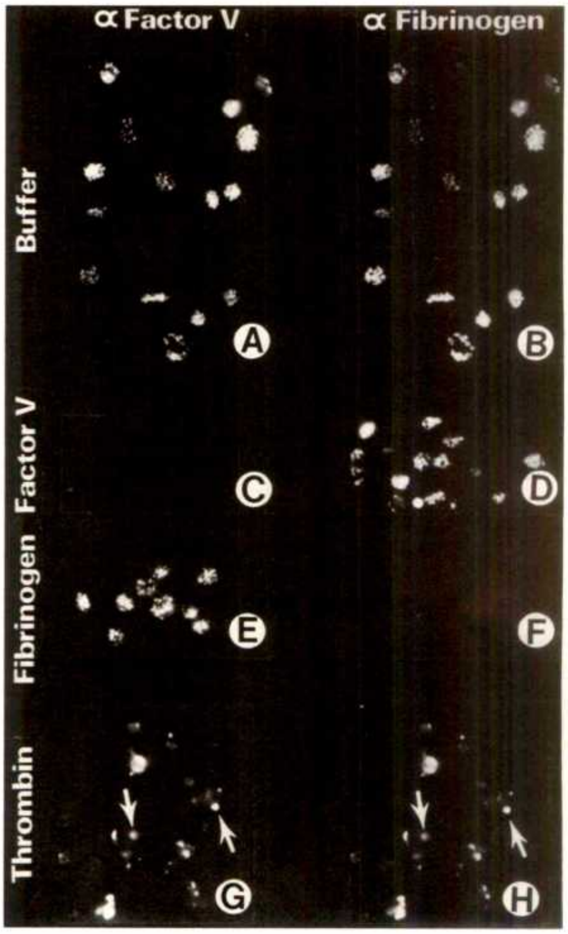

Fig 2. Colocalization of Factor V and Fbg in resting and thrombin-stimulated platelets. Resting, permeable platelets were stained with a mixture of rabbit antihuman Factor V and rhodaminated goat $F(ab')_2$ antihuman Fbg and counterstained with fluoresceinated goat $F(ab')_2$ antirabbit IgG. Antibodies absorbed with buffer alone (A and B) demonstrated punctate intracellular staining for Factor V which exactly colocalized with Fbg. Incubation of the antibody mixture prior to staining with either purified Factor V (C and D) or Fbg (E and F) only blocked fluorescence when an antibody was absorbed with its parent antigen. When platelets were fixed one minute following thrombin stimulation, we noted the appearance of large, peripheral immunofluorescent masses which are defined as smooth fluorescent structures ≥ 0.5 μm in diameter and which double stained for Factor V and Fbg (G and H, arrowheads); Original magnification, ×1,400; Current magnification, ×1,050.

In the article by Gelinas et al appearing in the June 1986 issue (Volume 68, pp 1777–1779), the publisher introduced an error in the title. The correct title is: Chinese $^{A}\gamma$ HPFH: C to T Substitution at Position −196 of the $^{A}\gamma$ Gene Promoter.

ANNOUNCEMENTS

AMERICAN SOCIETY OF HEMATOLOGY
TWENTY-EIGHTH ANNUAL MEETING
San Francisco
December 6–9, 1986
SUMMARY OF PROGRAM

Saturday, December 6
Morning: EDUCATION PROGRAM
- Hemoglobinopathies
- Acute Leukemia
- Myeloma
- Acquired Abnormalities in Platelet Function
- Pediatric Hematology
- Human Retrovirus Infection
- Molecular Genetics
- Hodgkins Disease
- Bone Marrow Transplantation
- Thrombosis
- Transfusion Medicine
- Biological Response Modifiers
- Immune Markers in the Diagnosis and Treatment of Hematopoietic Malignancies

Afternoon: EDUCATION PROGRAM
- Non-Hodgkins Lymphoma
- Acquired Abnormalities in Platelet Function
- Molecular Genetics
- The Chernobyl Disaster: Reflections on the Accident, The Problems of a Nuclear Accident Today and Are We Prepared
- Oncogenes in Hematopoietic Neoplasms
- Hemoglobinopathies
- Ham Wasserman Lecture: The World Distribution, Population Genetics, and Prevention of the Major Hemoglobin Disorders
- Acute Leukemia
- Myeloma
- Pediatric Hematology

SCIENTIFIC SUBCOMMITTEE SYMPOSIA
- Subcommittee on Hemoglobin/Red Cell
- Subcommittee on Pediatric Hematology
- Subcommittee on Transfusion Medicine
- Subcommittee on Leukocyte Physiology

FORUM ON THE PRACTICE OF HEMATOLOGY

SYMPOSIUM ON AIDS

Evening: Welcome Reception

Sunday, December 7
Morning: EDUCATION PROGRAM
- Hodgkins Disease
- Human Retrovirus Infection
- Biological Response Modifiers
- New Techniques in Education
- Non-Hodgkins Lymphoma
- Bone Marrow Transplantation
- Thrombosis
- Transfusion Medicine
- Oncogenes in Hematopoietic Neoplasms
- Immune Markers in the Diagnosis and Treatment of Hematopoietic Malignancies

SCIENTIFIC SUBCOMMITTEE SYMPOSIA
- Subcommittee on Neoplasia
- Subcommittee on Hemostasis
- Subcommittee on Hematopoietic Growth Factors
- Subcommittee on Immunohematology

SYMPOSIUM ON FLOW CYTOMETRY

Afternoon:
- FIRST BUSINESS MEETING
- PRESIDENT'S REPORT TO THE SOCIETY
- STRATTON LECTURE: RADICALS IN THE DEFENSE DEPARTMENT—SUPEROXIDE AND THE RESPIRATORY BURST
- **PLENARY SESSIONS**

Monday, December 8
Morning
- Simultaneous Scientific Sessions
- Scientific Poster Sessions

Afternoon
- Simultaneous Scientific Sessions
- Scientific Poster Sessions

Tuesday, December 9
Morning
- PRESIDENTIAL SYMPOSIUM: TRANSFER OF GENES
- GENERAL BUSINESS MEETING

Afternoon
- Simultaneous Scientific Sessions

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RESEARCH AND CLINICAL TRAINEE

A Research and Clinical Trainee position is available for MD training in hematology/oncology and bone marrow transplantation. Research project in leukemia, BMT, autoimmunity, or cell metabolism. Apply to Dr B.A. Cooper, Room A2.02, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1.

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THE XI CONGRESS OF THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTASIS

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Joint meetings with the European Society of Cardiology (President, P.G. Hugenholtz; Secretary-General, H. Kulbertus), July 7–8, 1987; with the International Society of Blood Transfusion (President, L. Barker; Chairman, A. André; Secretary-General, C. Vermylen), July 9–10, 1987; and with the World Federation of Haemophilia (President, F. Schnabel), July 9, 1987. In addition to free communication, posters, and poster symposia, there will be two Plenary Session Lectures and four State of the Art Lectures daily. The latter are authoritative, comprehensive overviews on a specific topic, especially designed for an audience not actively engaged in research in this particular field. At noon a Lunch Session on Novel Techniques and Methodology is organized in collaboration with the biomedical industry. President of the Congress: Professor M. Verstraete; Secretary-General: H.R. Lijnen, PhD. For further information, return form below to the Center for Thrombosis and Vascular Research, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Please print or type:

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Would you be interested in an I.T. (Inclusive Tour) ticket covering air transportation and hotel (with fixed dates of arrival and departure)? ________ yes ________ no

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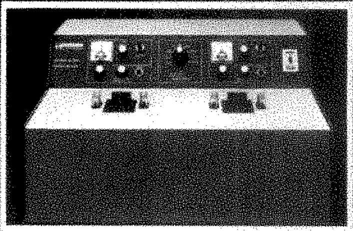

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For further information and for applications, contact:

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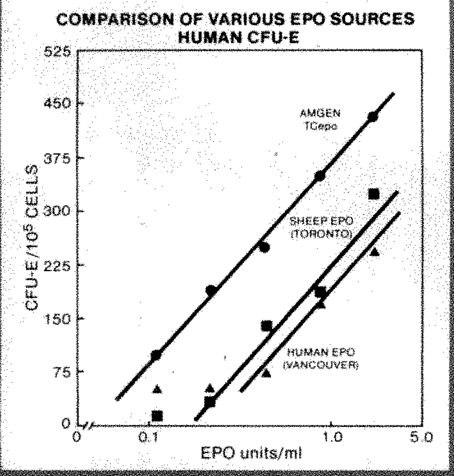
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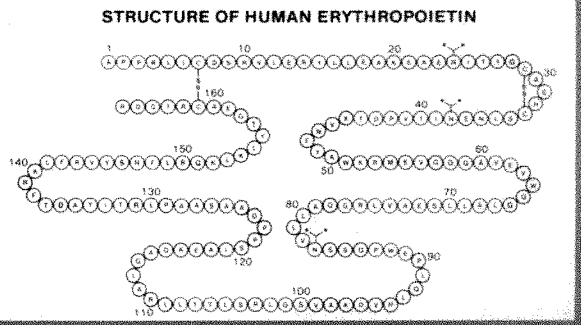
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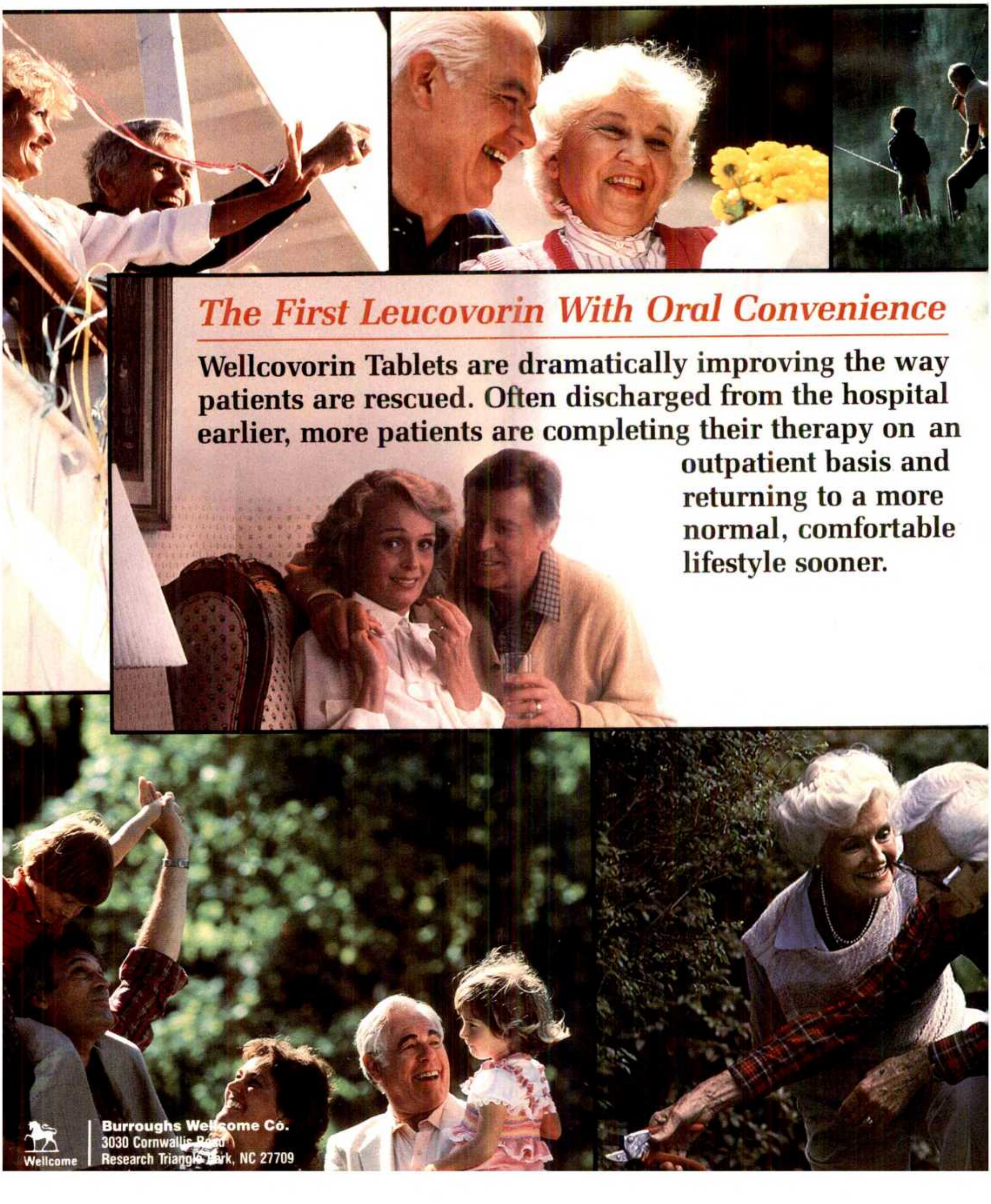

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CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer,* *41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med,* 68:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol,* *40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood,* *55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis,* *135*(3): 493-496, 1977.

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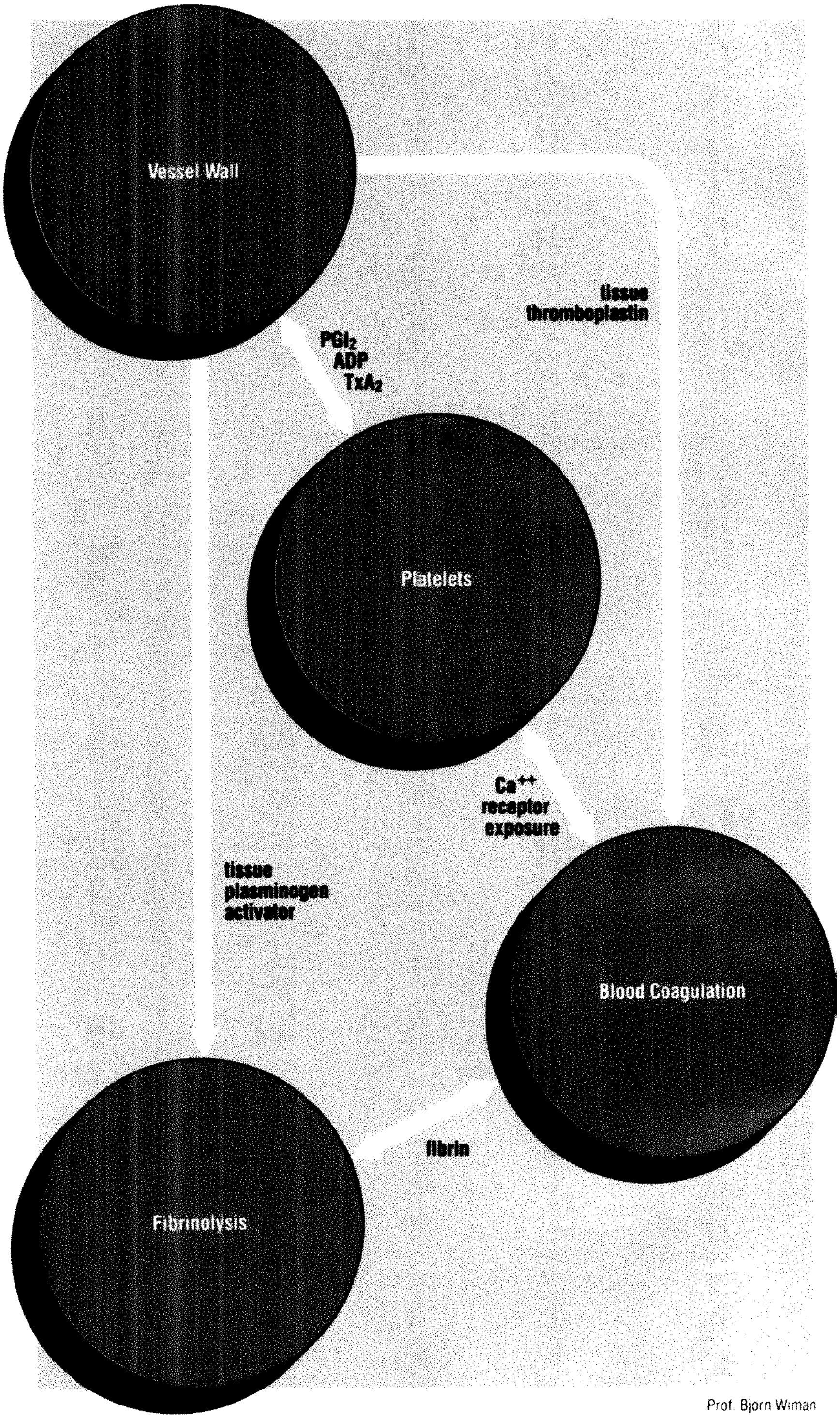

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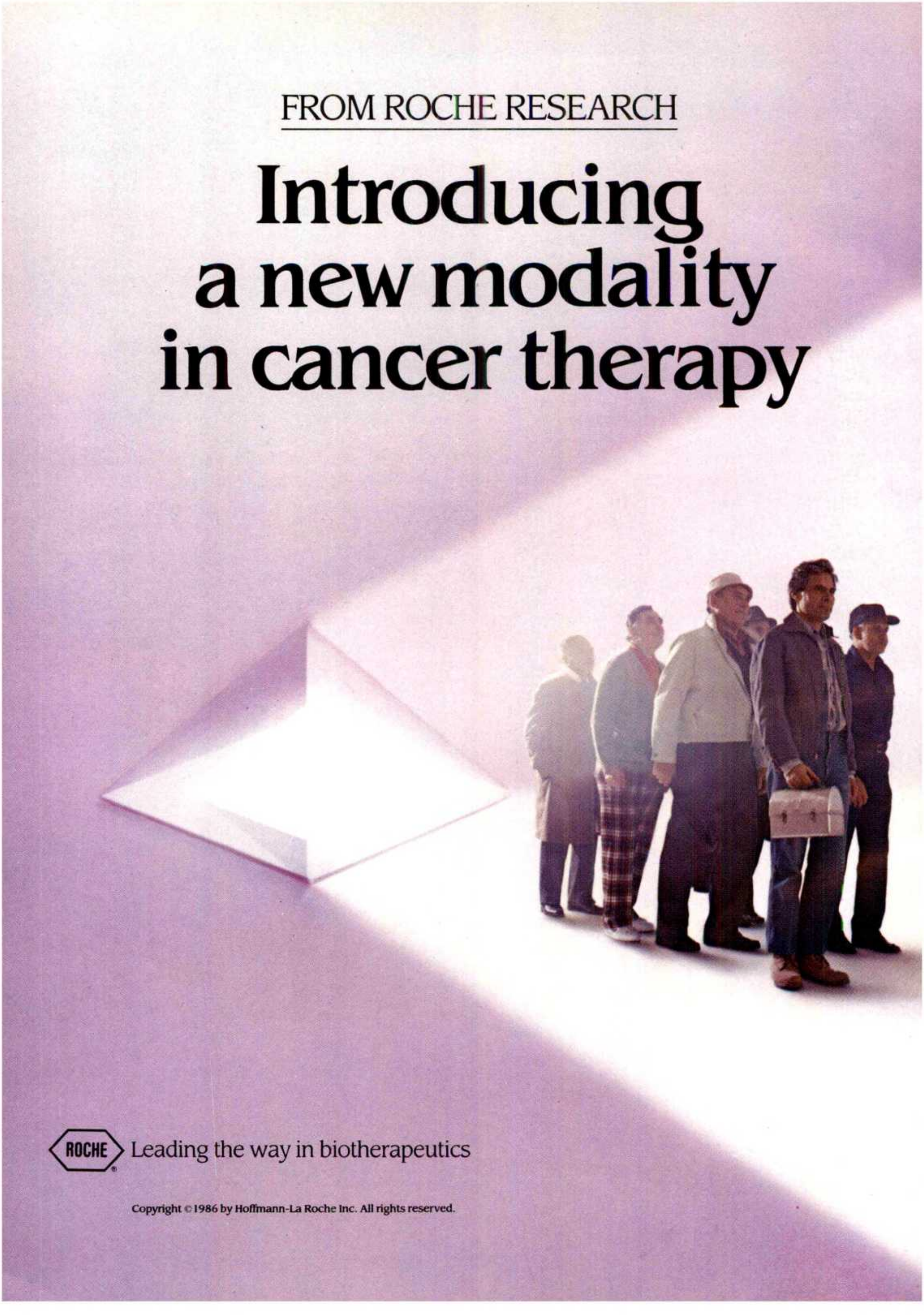

FROM ROCHE RESEARCH
Introducing a new modality in cancer therapy
ROCHE Leading the way in biotherapeutics

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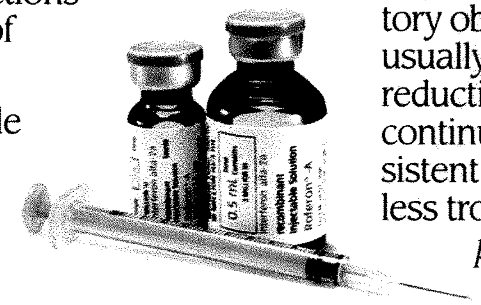
a breakthrough in hairy cell leukemia

- ☐ Effective in all stages of the disease.
- ☐ Statistically increased survival compared to a historical control group.
- ☐ Normalizes peripheral blood elements.
- ☐ Improves quality of life.
- ☐ Dramatically decreases the need for red blood cell and platelet transfusions.
- ☐ Virtually eliminates life-threatening opportunistic infections after several months of therapy.
- ☐ Ready-to-use injectable solution. No mixing or reconstitution necessary.
- ☐ SC or IM injection and fixed dosing permit administration without weight or body surface area calculation in home, office or institution.
- ☐ Caution patients not to change brands of Interferon without medical consultation, as a change in dosage may result.
- ☐ Common initial side effects, such as fever, chills, myalgia, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug. Persistent fatigue is found to be less troublesome with *p.m.* or *h.s.* administration. See following pages for details on all adverse reactions.



Injectable Solution

3 million IU unit-dose vial (3 million IU/mL)	18 million IU multidose vial (3 million IU/0.5 mL)

NEW SC/IM

ROFERON®-A

Interferon alfa-2a, recombinant/Roche

a promise fulfilled

Before prescribing, please see complete product information on following pages.

NEW SC/IM

ROFERON®-A

BRAND OF
Interferon alfa-2a, recombinant/Roche

DESCRIPTION: Roferon®-A (Interferon alfa-2a, recombinant/Roche) is a sterile protein product for use by injection. Roferon-A is manufactured by recombinant DNA technology that employs a genetically engineered *E. coli* bacterium containing DNA that codes for the human protein. Interferon alfa-2a, recombinant/Roche is a highly purified protein containing 165 amino acids, and it has an approximate molecular weight of 19,000 daltons. The purification procedure includes affinity chromatography using a murine monoclonal antibody. Roferon-A is supplied as an injectable solution. The solution is colorless and contains Interferon alfa-2a, recombinant/Roche, phenol as a preservative, sodium chloride for isotonicity and human serum albumin as a stabilizer. Fermentation is carried out in a defined nutrient medium containing the antibiotic tetracycline hydrochloride, 5 mg/liter. However, the presence of the antibiotic is not detectable in the final product.

Each vial of Roferon-A contains 3 million or 18 million international units (IU) of Interferon alfa-2a, recombinant/Roche. The specific activity of Interferon alfa-2a, recombinant/Roche, is 2×10^8 IU/mg protein. The route of administration is subcutaneous or intramuscular.

CLINICAL PHARMACOLOGY: The mechanism by which Interferon alfa-2a, recombinant/Roche, or any other interferon, exerts antitumor activity is not clearly understood. However, it is believed that direct antiproliferative action against tumor cells and modulation of the host immune response play important roles in the antitumor activity.

The biological activities of Interferon alfa-2a, recombinant/Roche are species-restricted, *i.e.*, they are expressed in a very limited number of species other than humans. As a consequence, preclinical evaluation of Interferon alfa-2a, recombinant/Roche has involved *in vitro* experiments with human cells and some *in vivo* experiments.[1] Using human cells in culture, Interferon alfa-2a, recombinant/Roche has been shown to have antiproliferative and immunomodulatory activities that are very similar to those of the mixture of interferon alfa subtypes produced by human leukocytes. *In vivo*, Interferon alfa-2a, recombinant/Roche has been shown to inhibit the growth of several human tumors growing in immunocompromised (nude) mice. Because of its species-restricted activity, it has not been possible to demonstrate antitumor activity in immunologically intact syngeneic tumor model systems, where effects on the host immune system would be observable. However, such antitumor activity has been repeatedly demonstrated with, for example, mouse interferon-alfa in transplantable mouse tumor systems. The clinical significance of these findings is unknown.

The metabolism of Interferon alfa-2a, recombinant/Roche is consistent with that of alfa interferons in general. Alfa interferons are totally filtered through the glomeruli and undergo rapid proteolytic degradation during tubular reabsorption, rendering a negligible reappearance of intact alfa interferon in the systemic circulation. Small amounts of radiolabeled Interferon alfa-2a, recombinant/Roche appear in the urine of isolated rat kidneys, suggesting near complete reabsorption of Interferon alfa-2a, recombinant/Roche catabolites. Liver metabolism and subsequent biliary excretion are considered minor pathways of elimination for alfa interferons.

The serum concentrations of Interferon alfa-2a, recombinant/Roche reflected a large intersubject variation in both healthy volunteers and patients with disseminated cancer.

In healthy people, Interferon alfa-2a, recombinant/Roche exhibited an elimination half-life of 3.7 to 8.5 hours (mean 5.1 hours), volume of distribution at steady-state of 0.223 to 0.748 L/kg (mean 0.400 L/kg) and a total body clearance of 2.14 to 3.62 mL/min/kg (mean 2.79 mL/min/kg) after a 36 million IU (2.2×10^8 pg) intravenous infusion. After intramuscular and subcutaneous administrations of 36 million IU,peak serum concentrations ranged from 1500 to 2580 pg/mL (mean 2020 pg/mL) at a mean time to peak of 3.8 hours and from 1250 to 2320 pg/mL (mean 1730 pg/mL) at a mean time to peak of 7.3 hours, respectively. The apparent fraction of the dose absorbed after intramuscular injection was greater than 80%.

The pharmacokinetics of Interferon alfa-2a, recombinant/Roche after single intramuscular doses to patients with disseminated cancer were similar to those found in healthy volunteers. Dose proportional increases in serum concentrations were observed after single doses up to 198 million IU. There were no changes in the distribution or elimination of Interferon alfa-2a, recombinant/Roche during twice daily (0.5 to 36 million IU), once daily (1 to 54 million IU), or three times weekly (1 to 136 million IU) dosing regimens up to 28 days of dosing. Multiple intramuscular doses of Interferon alfa-2a, recombinant/Roche resulted in an accumulation of 2 to 4 times the single dose serum concentrations. Pharmacokinetic information in patients with hairy cell leukemia is presently unknown.

The acute parenteral toxicity of Interferon alfa-2a, recombinant/Roche has been studied in mice, rats, rabbits and ferrets at doses up to 30 million IU/kg intravenously, and 500 million IU/kg intramuscularly. No treatment-related mortality was noted in any species given Interferon alfa-2a, recombinant/Roche by any of the routes of administration.

Effects on Hairy Cell Leukemia: During the first one to two months of treatment of patients with hairy cell leukemia, significant depression of hematopoiesis was likely to occur. Subsequently, there was improvement in circulating blood cell counts.

Of the 75 patients who were evaluable for efficacy following at least 16 weeks of therapy, 46 (61%) achieved complete or partial response. Twenty-one patients (28%) had a minor remission, eight (11%) remained stable, and none had worsening of disease. All patients who achieved either a complete or partial response had complete or partial normalization of all peripheral blood elements including hemoglobin level, white blood cell, neutrophil, monocyte and platelet counts with a concomitant decrease in peripheral blood and bone marrow hairy cells. Responding patients also exhibited a marked reduction in red blood cell and platelet transfusion requirements, a decrease in infectious episodes and improvement in performance status. The probability of survival for two years in patients receiving Roferon-A (94%) was statistically increased compared to a historical control group (75%).

ROFERON®-A (Interferon alfa-2a, recombinant/Roche)

INDICATIONS AND USAGE: Roferon-A is indicated for use in the treatment of hairy cell leukemia in people 18 years of age or older. Studies have shown that Roferon-A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and nonsplenectomized patients.[2,3]

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically (*e.g.*, monthly) during treatment to determine whether response to treatment has occurred. If a patient does not respond within six months, treatment should be discontinued. If a response to treatment does occur, treatment should be continued until no further improvement is observed and these laboratory parameters have been stable for about three months. It is not known whether continued treatment after that time is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS: Roferon-A is contraindicated in patients with known hypersensitivity to alfa interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Roferon-A should be administered under the guidance of a qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of the therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function.

Because of the possibility of severe or even fatal adverse reactions, patients should be informed not only of the benefits of therapy but also of the risks involved.

Roferon-A should be administered with caution to patients with cardiac disease or with any history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (*i.e.*, fever, chills) frequently associated with Roferon-A administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred in patients receiving Roferon-A.

Caution should be exercised when administering Roferon-A to patients with myelosuppression.

Central nervous system adverse reactions have been reported in a number of patients. These reactions included decreased mental status, exaggerated central nervous system function, and dizziness. More severe obtundation and coma have been rarely observed. Most of these abnormalities were mild and reversible within a few days to three weeks upon dose reduction or discontinuation of Roferon-A therapy. Careful periodic neuropsychiatric monitoring of all patients is recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* In all instances where the use of Roferon-A is considered for chemotherapy, the physician must evaluate the need and usefulness of the drug against the risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken according to the clinical judgment of the physician. Reinstitution of Roferon-A therapy should be carried out with caution and with adequate consideration of the further need for the drug and alertness as to possible recurrence of toxicity.

The minimum effective dose of Roferon-A for treatment of hairy cell leukemia has not been established.

Information for Patient: Patients should be cautioned not to change brands of Interferon without medical consultation, as a change in dosage may result. Patients should be informed regarding the potential benefits and risks attendant to the use of Roferon-A. If home use is determined to be desirable by the physician, instructions on appropriate use should be given, including review of the contents of the enclosed Patient Information Sheet. Patients should be well hydrated, especially during the initial stages of treatment.

Laboratory Tests: Periodic complete blood counts and liver function tests should be performed during the course of Roferon-A treatment. They should be performed prior to therapy and at appropriate periods during therapy. Since responses of hairy cell leukemia are not generally observed for one to three months after initiation of treatment, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase of treatment.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility:

Carcinogenesis: Roferon-A has not been tested for its carcinogenic potential.

Mutagenesis: A. Internal studies—Ames tests using six different tester strains, with and without metabolic activation, were performed with Roferon-A up to a concentration of 1920 µg/plate. There was no evidence of mutagenicity.

Human lymphocyte cultures were treated *in vitro* with Roferon-A at noncytotoxic concentrations. No increase in the incidence of chromosomal damage was noted.

B. Published studies—There are no published studies on the mutagenic potential of Roferon-A. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported.

A chromosomal defect following the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder has been reported.

In contrast, other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon.

It has also been shown that human leukocyte interferon protects primary chick embryo fibroblasts from chromosomal aberrations produced by gamma rays.

Impairment of Fertility: Roferon-A has been studied for its effect on fertility in Macaca mulatta (rhesus monkeys). Nonpregnant rhesus females treated with Roferon-A at doses of 5 and 25 million IU/kg/day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory. These monkeys returned to a normal menstrual rhythm following discontinuation of treatment.

Drug Interactions: Interactions between Roferon-A and other drugs have not been fully evaluated.

PREGNANCY: *Teratogenic Effects:* Pregnancy Category C. Roferon-A has been shown to demonstrate a statistically significant increase in abortifacient activity in rhesus monkeys when given at approximately 20 to 500 times the human dose. A study in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation) has failed to demonstrate teratogenic activity for Roferon-A. There are no adequate and well-controlled studies in pregnant women.

Nonteratogenic Effects: Dose-related abortifacient activity was observed in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg/day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation). A late-fetal period study (days 79 to 100 of gestation) is in progress and as yet there have been no reports of any increased rate of abortion.

Usage in Pregnancy: Safe use in human pregnancy has not been established. Therefore, Roferon-A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Information from primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during the therapy period.

Male fertility and teratologic evaluations have yielded no significant adverse effects to date.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Roferon-A, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use: Safety and effectiveness in children under 18 years of age have not been established.

ADVERSE REACTIONS: The following data on adverse reactions are based on the subcutaneous or intramuscular administration of Roferon-A as a single agent for the treatment of 63 patients with hairy cell leukemia during investigational trials in the United States.

Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%) and headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), dryness or inflammation of the oropharynx (16%), dry skin or pruritus (13%) and weight loss (14%) were observed with moderate frequency. Less commonly, diaphoresis (8%), paresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of herpes labialis (8%), transient impotence (6%) and arthralgias (5%) were also observed. Rarely (<3%), central nervous system effects including decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias and palpitations, were reported.

Adverse experiences which occurred rarely and may have been related to underlying disease, included epistaxis, bleeding gums, ecchymosis and petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis and inflammation at the site of injection, were also rarely observed.

Roferon-A has also been evaluated for the treatment of many other types of cancer under investigational trials in the United States. These studies generally utilized higher doses (12 to 50 million IU/m^2). All of the previously described adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in patients who received higher doses of Roferon-A. Some additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%) and edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: *Gastrointestinal*—abdominal fullness, hypermotility and hepatitis; *Central Nervous System*—gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia and loss of libido; *Peripheral Nervous System*—muscle contractions; *Cardiovascular*—congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon and hot flashes; *Pulmonary*—bronchospasm and tachypnea; *Miscellaneous*—excessive salivation and flushing of skin.

Abnormal Laboratory Test Values: The percentage of patients with hairy cell leukemia or with other types of malignancies who experienced a significant abnormal laboratory test value at least once during their treatment is shown in the following table:

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary[*] (n = 1019)
Hematologic		
Leukopenia	59%	69%
Neutropenia	39%	58%
Thrombocytopenia	42%	42%
Decreased Hemoglobin	36%	6.3%
Decreased Hematocrit	43%	12.5%
Mean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
Hepatic		
SGOT	47%	78%
Alkaline Phosphatase	18%	48%
LDH	12%	47%
Bilirubin	1.6%	31%
Renal/Urinary		
BUN	2%	10%
Serum Creatinine	3%	10%
Uric Acid[†]	6%	15%
Proteinuria[†]	10%	25%
Other Chemistry Tests		
Hypocalcemia	10%	51%
Elevated FBS[‡]	33%	39%
Elevated Serum Phosphorus	2%	17%

[*] Patients with hairy cell leukemia are included in this overall summary.

[†] The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.

[‡] Random glucose determination.

Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.[4]

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count <50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended.

Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.

DIRECTIONS FOR USE: The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count <50,000) or who are at risk for bleeding.

Injectable Solution:

3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative.

18 million IU Roferon-A per vial (for multiple-dose use)—Each 1 mL contains 6 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride for isotonicity, 5 mg human serum albumin as a stabilizer and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche.

The injectable solution should be stored in the refrigerator at 36° to 46°F (2° to 8°C). Do *not* freeze. Do not shake.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

HOW SUPPLIED: Sterile Roferon-A is supplied as single and multiple-dose vials:

Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01).

Roferon-A Injectable Solution: Multiple-dose vials containing 18 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09).

REFERENCES:

1. Trown PW *et al: Cancer 57*(Suppl.):1648-1656, 1986.
2. Foon KA *et al: Blood 64*(Suppl. 1):164a, 1984.
3. Quesada JR *et al: Cancer 57*(Suppl.):1678-1680, 1986.
4. Jones GJ, Itri LM: *Cancer 57*(Suppl.):1709-1715, 1986.

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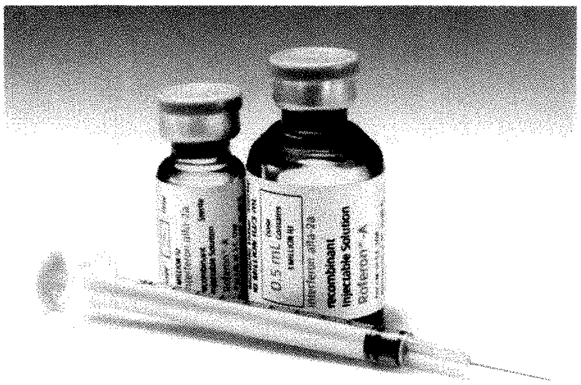


Roche Laboratories
Division of Hoffmann-La Roche Inc.
Nutley, New Jersey 07110

Today's IGIV. Don't settle for less.

For immunodeficiency syndromes: aggressive protection against infection

Patients without sufficient levels of antibodies face overwhelming infection, and they need help. Infusion with GAMIMUNE® N gives these patients the edge that may make the difference: antibodies against major bacterial, viral and fungal pathogens... in just minutes.

For both chronic and acute ITP in children and adults: rapid elevation of platelet levels

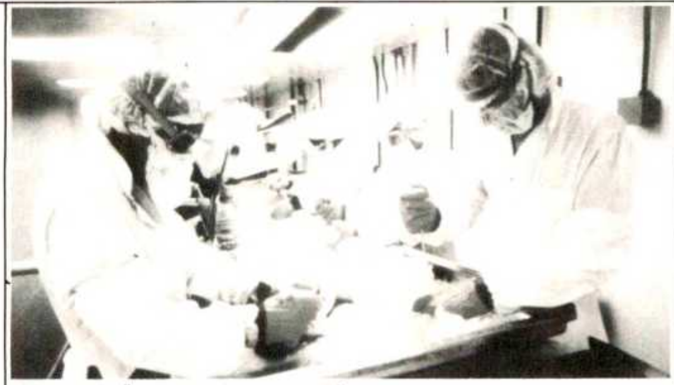
GAMIMUNE® N may initiate a therapeutic increase in platelets in both children and adults with idiopathic thrombocytopenic purpura (ITP).[1*†] Treatment with GAMIMUNE® N has enabled some ITP patients to undergo major surgery.[1]

IGIV that's at least 98% pure gamma globulin

Cutter's fractionation method (Cohn-Oncley 6 & 9) contains all the necessary steps to produce high purity immune globulin. GAMIMUNE® N contains no foreign contaminants, no detectable levels of protein aggregates or vasoactive enzymes.[2] With GAMIMUNE® N you get what you expect ...IGIV containing at least 98% pure gamma globulin.[1]

GAMIMUNE® N

Immune Globulin
Intravenous (Human) 5%
(in 10% Maltose), pH 4.25

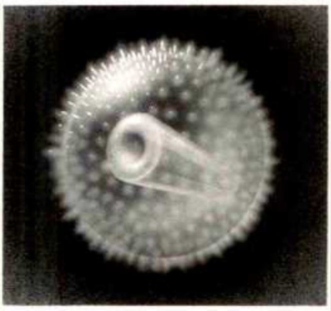


"Native" IGIV

GAMIMUNE® N is manufactured without chemical modification. This means GAMIMUNE® N antibodies maintain normal opsonization capacity, have a normal distribution of IgG subgroups and provide the immunologic activity of normal plasma.[1]

Documented in vitro inactivation of HTLV-III virus[‡]

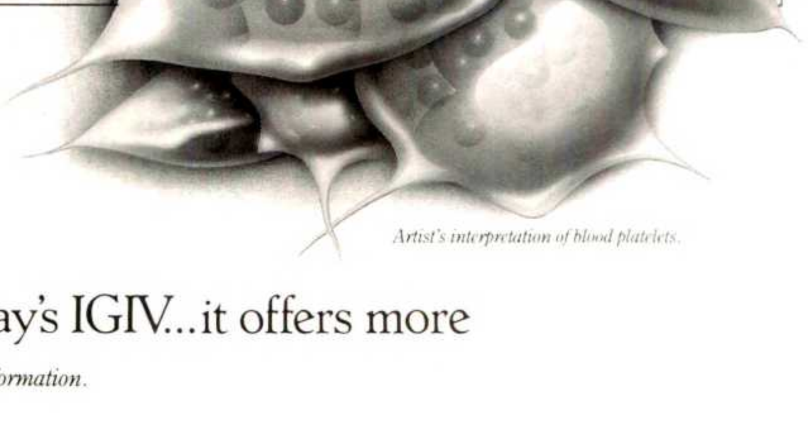
[‡] The Cohn-Oncley process used to produce GAMIMUNE®N has been challenged *in vitro* with a virus spike of 1 x 10^5 of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, GAMIMUNE®N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 reduction of HTLV-III.[3] In a separate study of intravenous immune globulins conducted by the Food and Drug Administration, an *in vitro* challenge at each of the six Cohn-Oncley fractionation steps using HTLV-III demonstrated a cumulative reduction of greater than 1 x 10^{15} infectious units.[4]



Artist's interpretation of HTLV-III virus.

Today's simple, economical IGIV: the only ready-to-use liquid

Dose for dose, GAMIMUNE® N saves money by eliminating valuable reconstitution time in the pharmacy. Gram for gram of protein, GAMIMUNE® N is affordable; there is no premium price for TODAY'S IGIV.



Artist's interpretation of blood platelets.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

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[*] It is not possible to predict which patients with ITP will respond to therapy.

[†] In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: *Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.*

Idiopathic Thrombocytopenic Purpura (ITP): *Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.*

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: *Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.*

Drug Interactions: *If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.*

Pregnancy Category C: *Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.*

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: *The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.*

Idiopathic Thrombocytopenic Purpura: *An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.*

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be ***gradually*** *increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.*

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. *GAMIMUNE® N Product License Application.* **2.** *Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. Rev Infect Dis 1986;8(Jul–Aug): S382–S390.* **3.** *Mitra G, Wong MF, Mosen MM, et al: Elimination of infectious retrovirus during preparation of immunoglobulins. Submitted for publication in Transfusion.* **4.** *Wells MA, Wittek AE, Epstein JS, et al: Inactivation and partition of human T-cell lymphotropic virus, type III, during ethanol fractionation of plasma. Transfusion 1986; 26(2): 210–213.*

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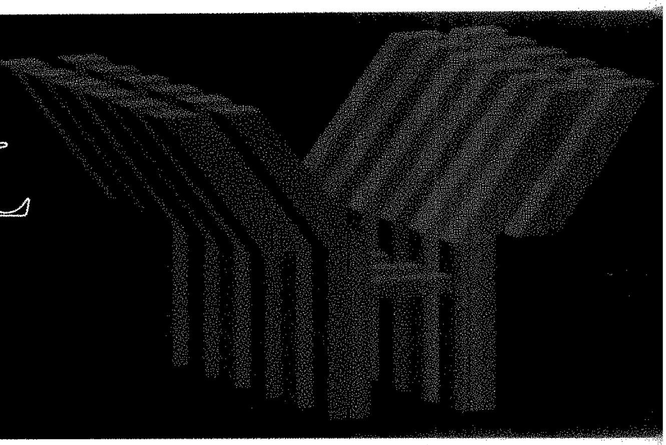

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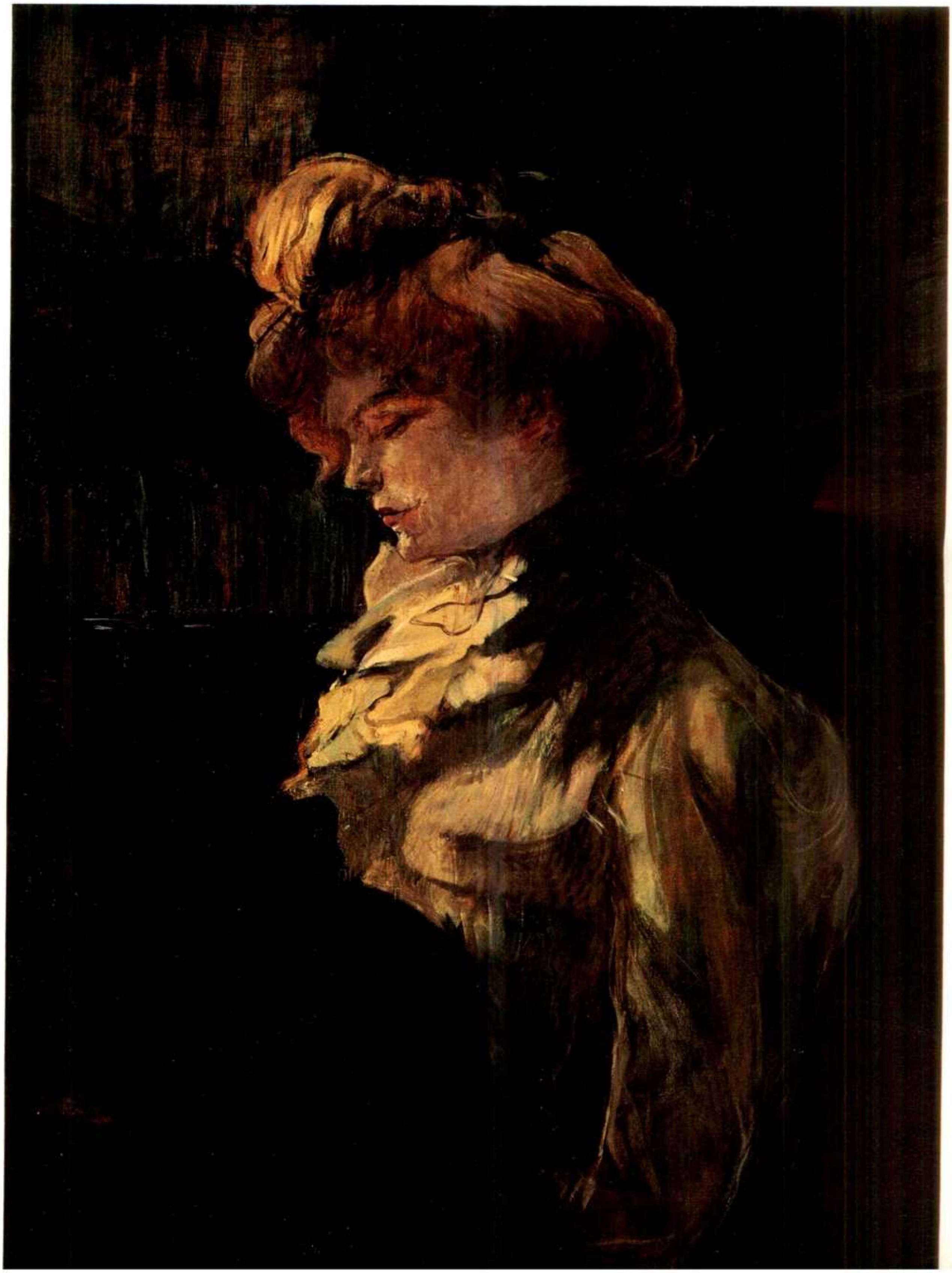

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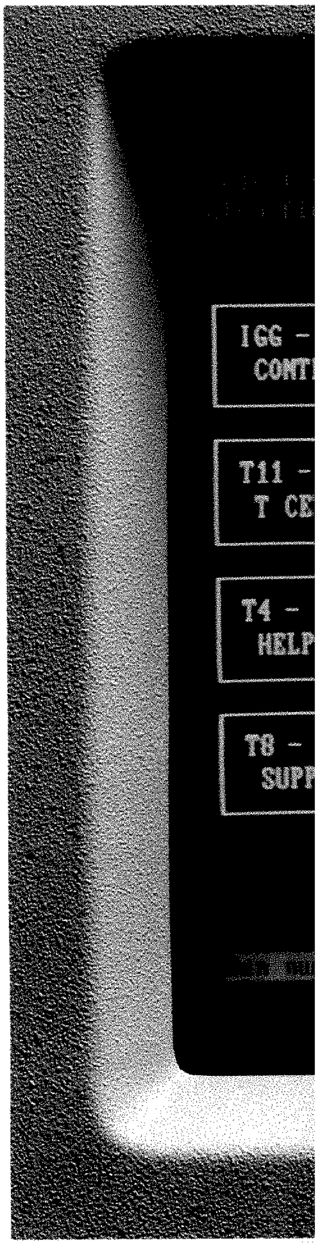

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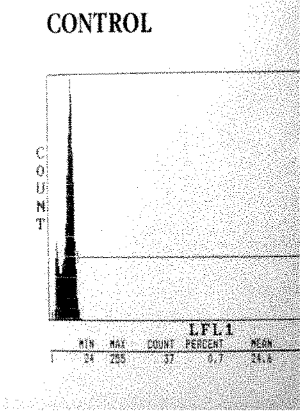

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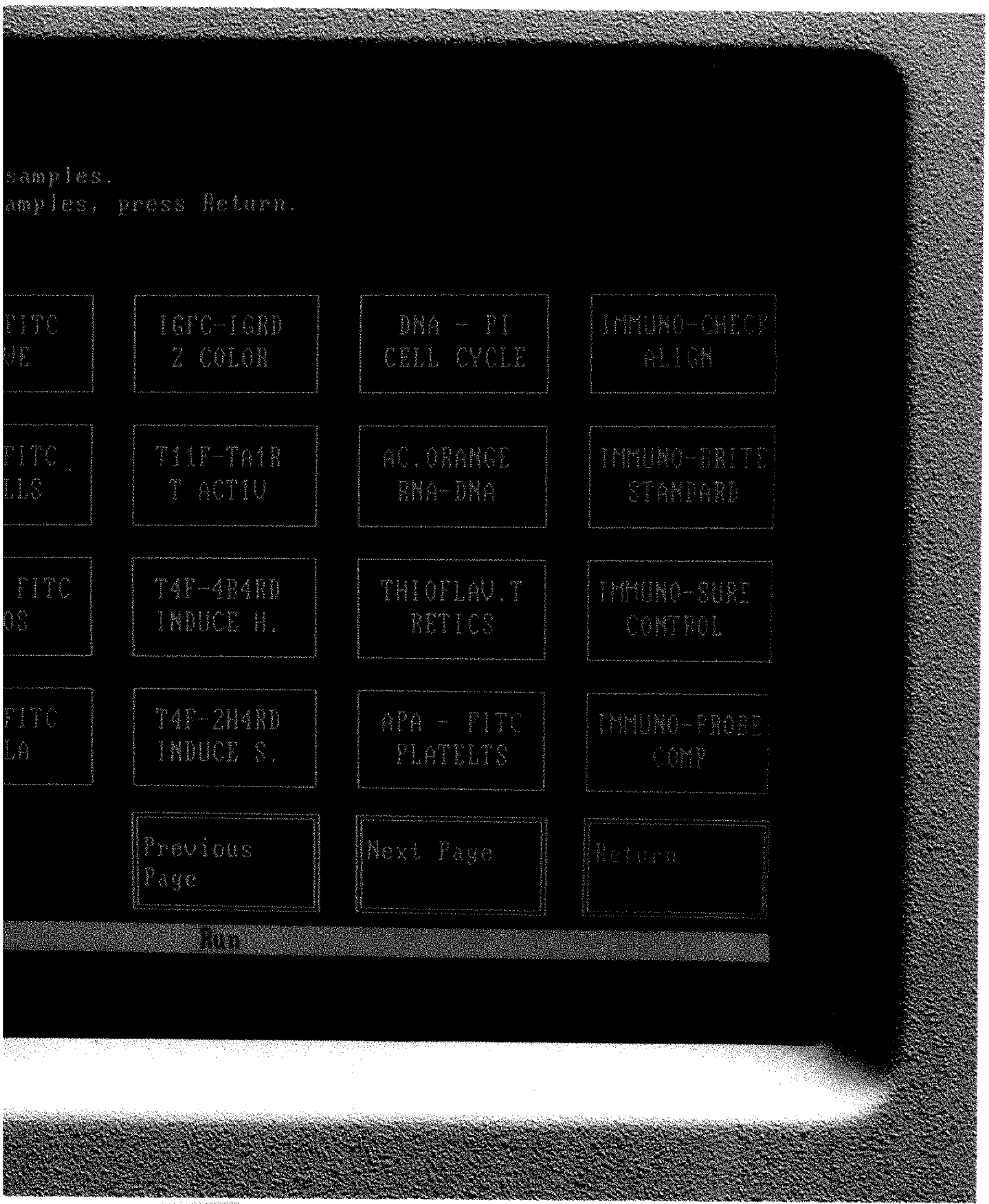

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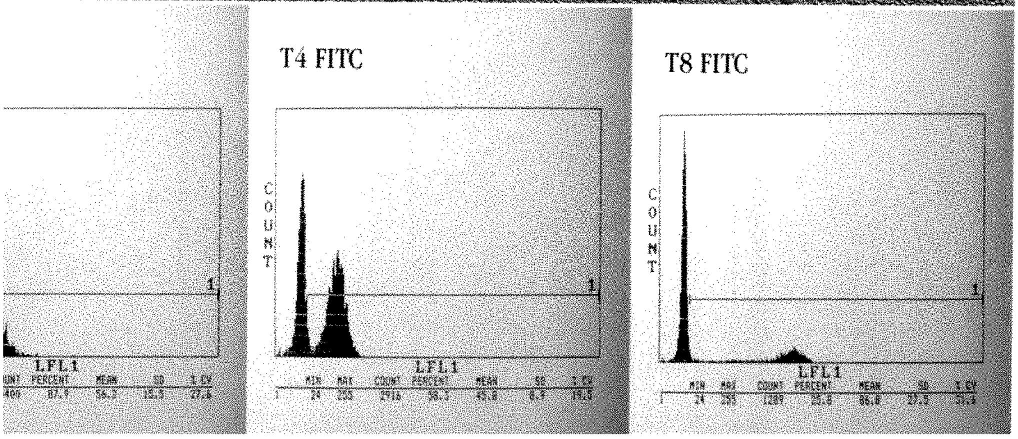

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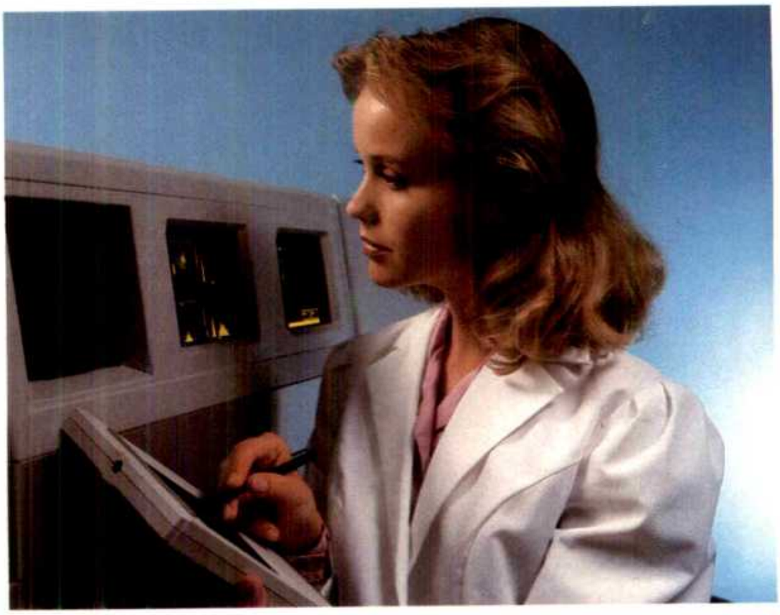

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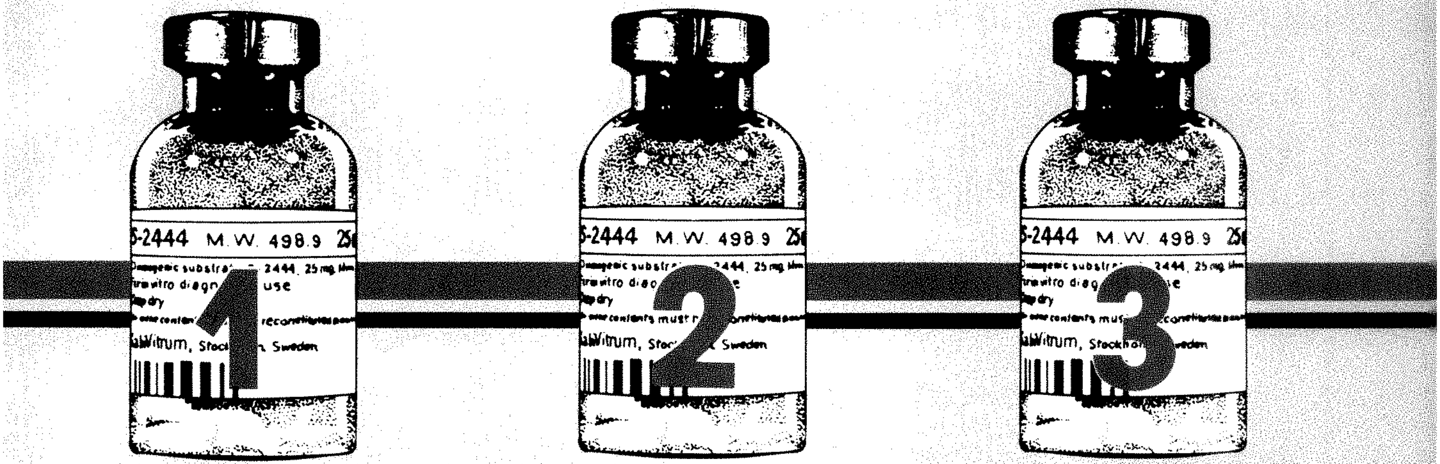

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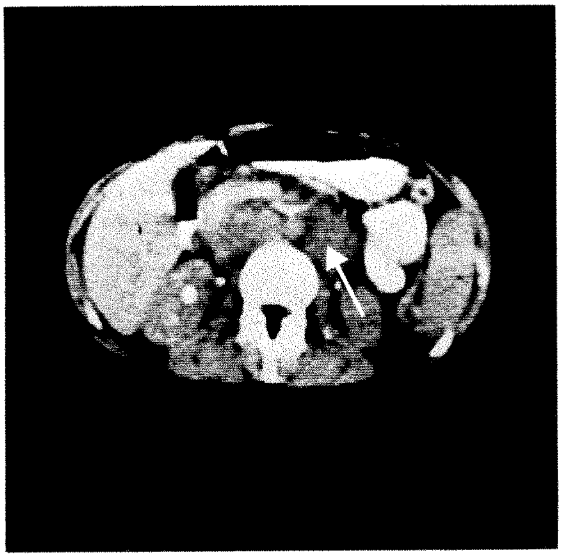

Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
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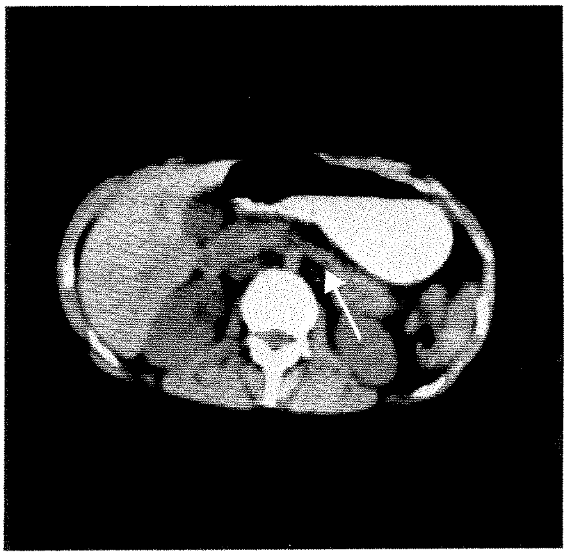

No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al:* Combination chemotherapy advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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M-BACOD

. Skarin AT, Canellos GP, Rosenthal DS, *et al*: Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.

. Anderson KC, Skarin AT, Rosenthal DS, *et al*: Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al*: Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), *m*-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

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Blenoxane® *(sterile bleomycin sulfate)*

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe.

Suggested preventive measures are:

(1) Maintain $FI\ O_2$ at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press:

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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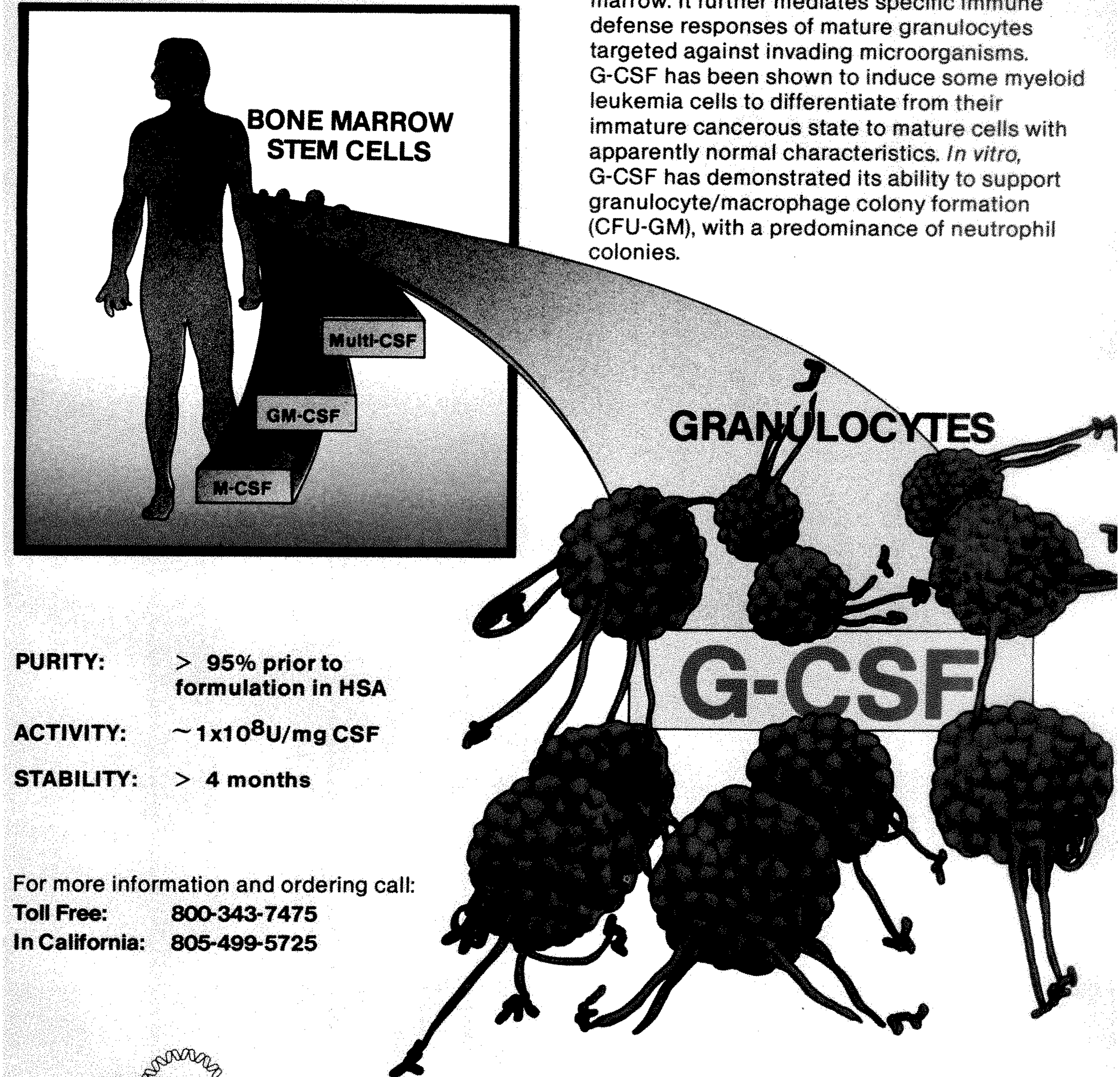

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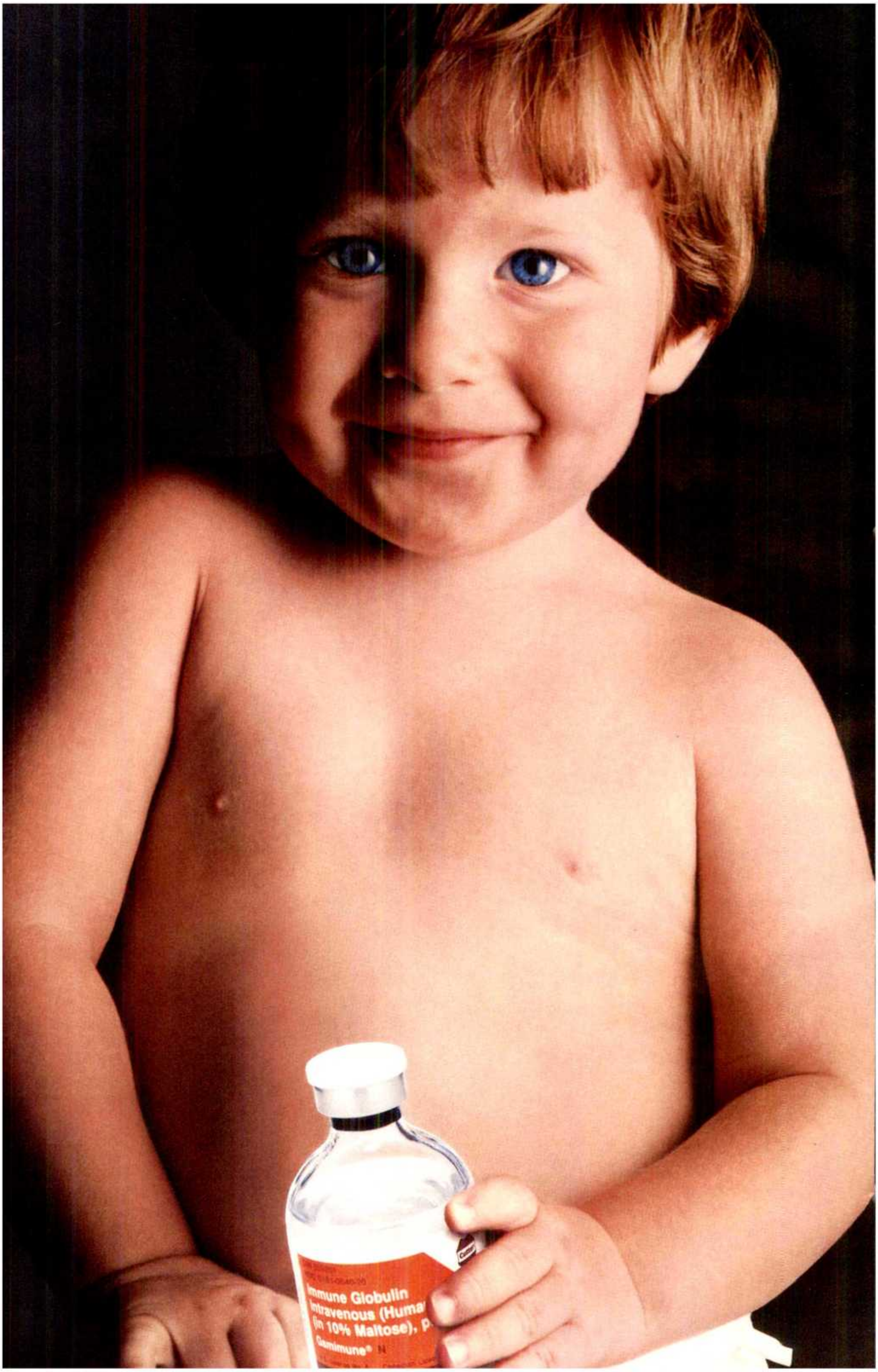
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In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

U.S. License No. 8

REFERENCES: 1. GAMIMUNE® N Package Insert. **2.** Mitra G, Wong MF, Mosen MM, et al: Elimination of infectious retrovirus during preparation of immunoglobulins. Submitted for publication in Transfusion. **3.** Wells MA, Wittek AE, Epstein JS, et al: Inactivation and partition of human T-cell lymphotropic virus, type III, during ethanol fractionation of plasma. Transfusion 1986; 26(2): 210–213.

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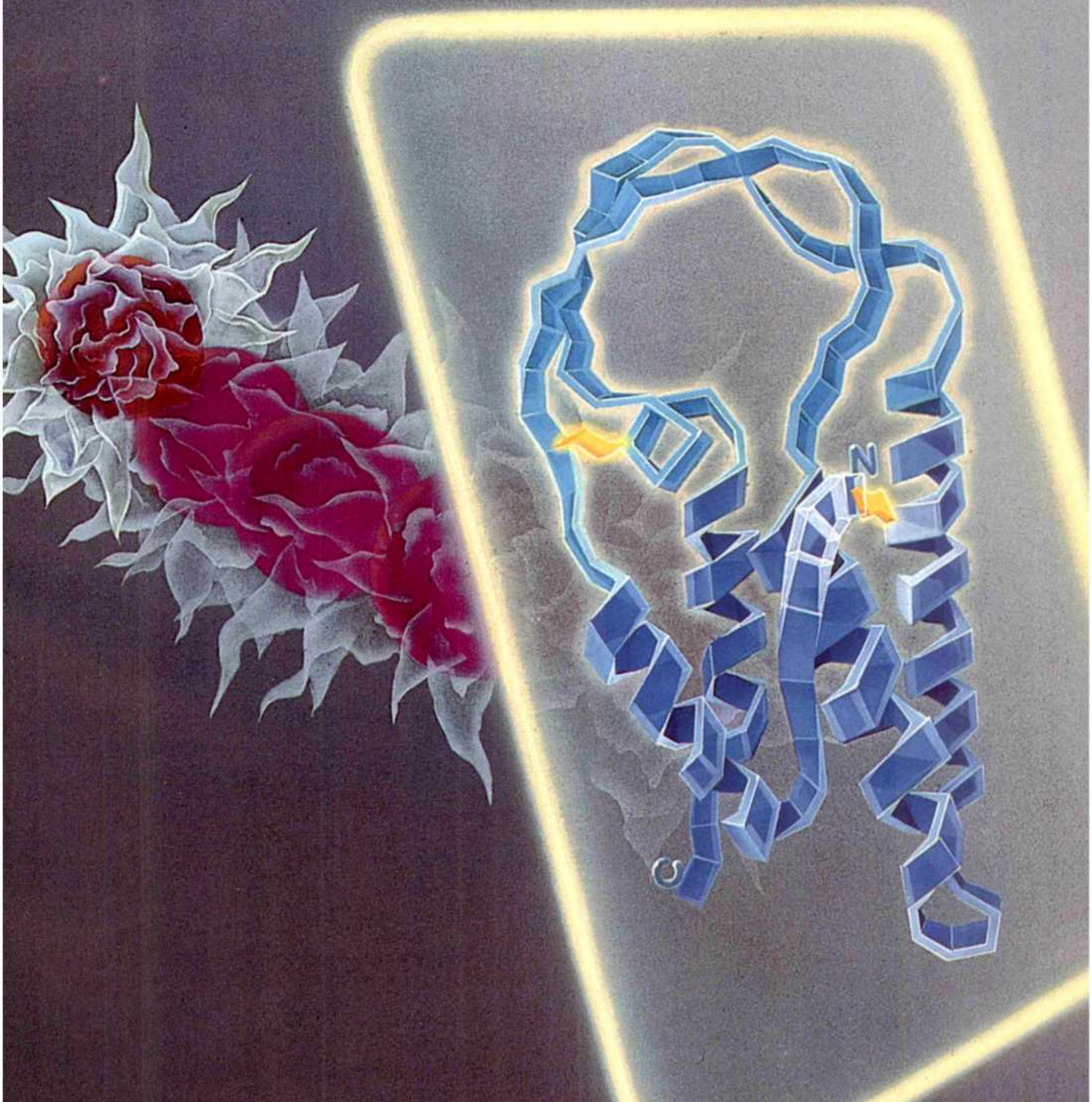
FROM ADVANCES IN BIOTECHNOLOGY...

NEW INTRON® A

INTERFERON ALFA-2b, RECOMBINANT INJECTION

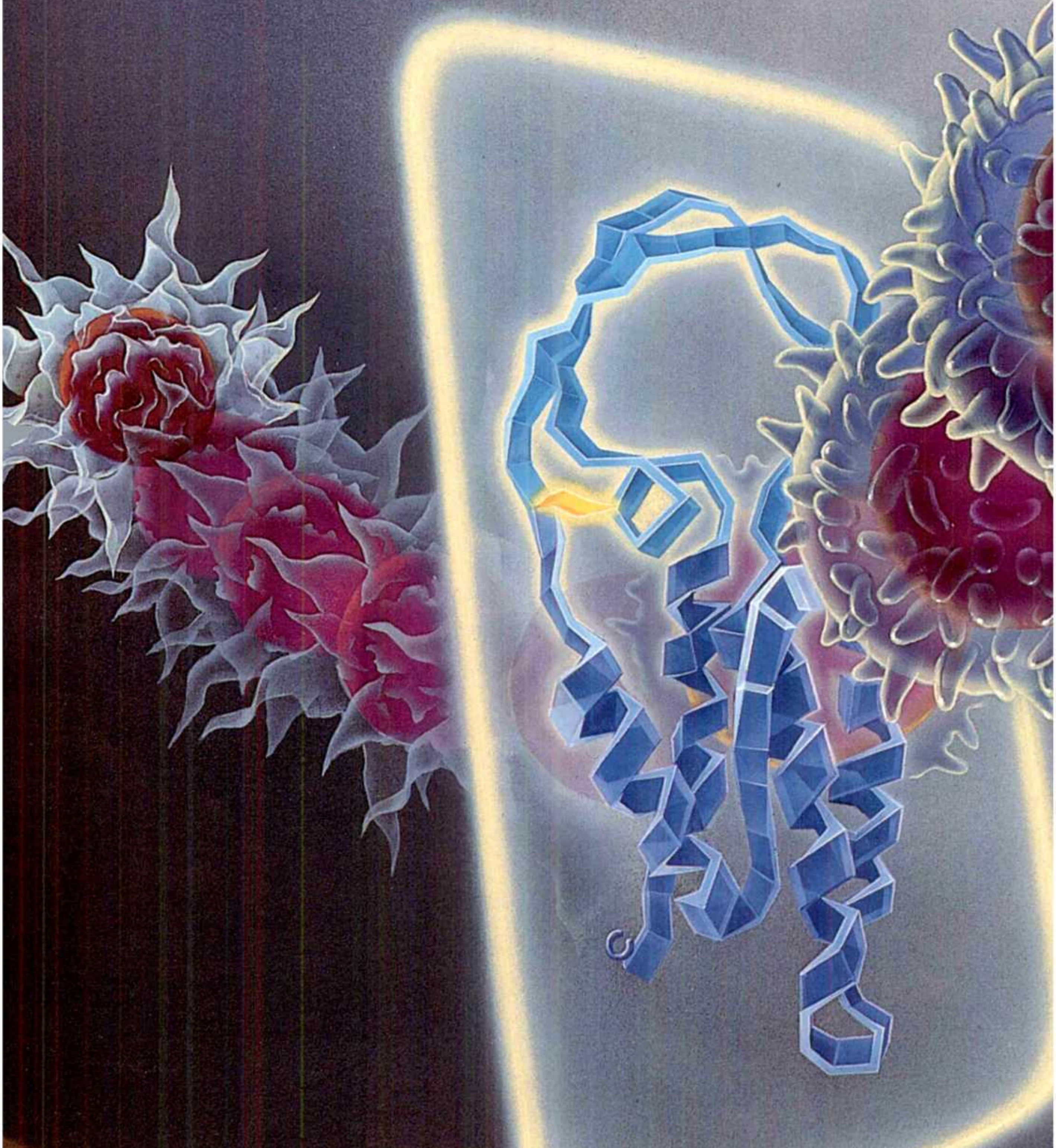
THE FIRST INTERFERON FROM SCHERING RESEARCH

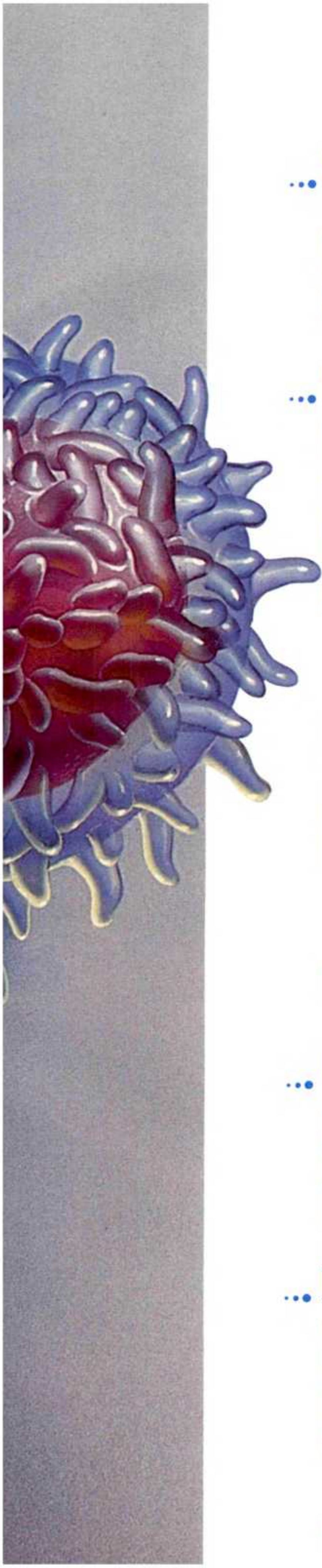


NEW INTRON® A

INTERFERON ALFA-2b, RECOMBINANT INJECTION

CHANGING THE COURSE OF HAIRY CELL LEUKEMIA





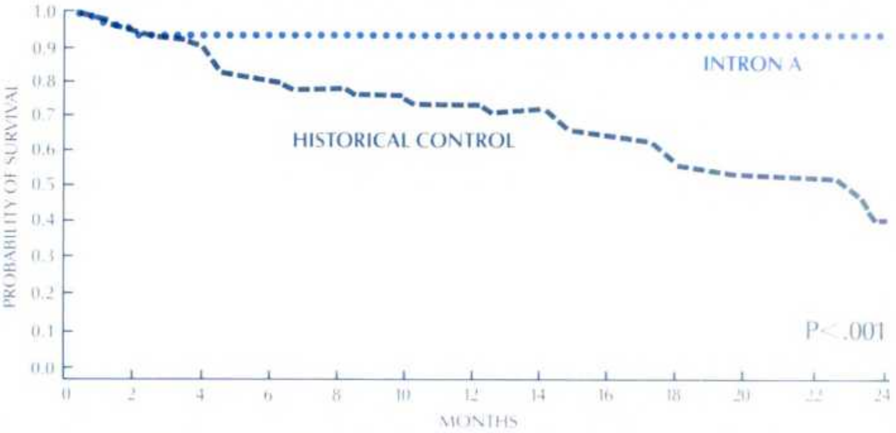
EFFICACY WELL ESTABLISHED

The efficacy of INTRON A was established in a 26-center clinical trial of 145 patients with progressive hairy cell leukemia, a B-cell malignancy.[1,2,3] INTRON A achieved a 74% objective response, measured as normalization of granulocyte, hemoglobin and platelet values and elimination of transfusion dependency.[1]

92% PROBABILITY OF SURVIVAL

In a separate analysis, a 24-month comparison was conducted between the INTRON A study population and historical controls.* After three months of therapy, patients receiving INTRON A reached and maintained a plateau of 92% probability of survival. The probability of survival among historical controls, however, declined steadily to 40% at 24 months.[1]

KAPLAN–MEIER ESTIMATED SURVIVAL CURVES



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*Seventy-one historical control patients were treated with current standard therapies, 85% with chemotherapy (mainly chlorambucil) and 15% with supportive care.

DRAMATIC CLINICAL IMPROVEMENTS

More than 80% of the study population achieved normal levels of platelets by three months, of hemoglobin by six months and of granulocytes by twelve months. Marked decreases in the incidence of life-threatening infections and transfusion dependency preceded normalization of hematologic values.[1]

THERAPY WELL TOLERATED

Treatment-related side effects were generally mild to moderate and rapidly reversible upon discontinuation of therapy. The predominant complaint was a flu-like syndrome, which usually diminished with continued therapy. Mild-to-moderate skin rashes occurred intermittently in 40% of the study population. A low incidence of mild-to-moderate GI disorders was also reported.[1]

For a brief summary of prescribing information, please see the following page.

NEW

INTRON® A

INTERFERON ALFA-2b, RECOMBINANT INJECTION

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A BIOLOGIC RESPONSE MODIFIER

To date, interferon actions in experimental systems have not been directly correlated with its clinical efficacy. However, as a biologic response modifier, interferon may modulate the immune system,[4,5,6] indirectly affect cell differentiation[7] and phenotypic expression[8,9] and enhance protection against viral infection.[10] In addition, interferon may have antiproliferative activity in experimental systems, seen as oncogene inhibition[11,12] and cytostatic action.[13,14,15]

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PRODUCT INFORMATION

THE FIRST INTERFERON FROM SCHERING RESEARCH

INTRON® A Interferon alfa-2b recombinant for Injection

BRIEF SUMMARY

INDICATIONS AND USAGE INTRON A for Injection is indicated for the treatment of patients 18 years of age or older with hairy cell leukemia. Studies have shown that INTRON A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and non-splenectomized patients.

Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically during treatment to determine whether response to treatment has occurred. If a patient does not respond within 6 months, treatment should be discontinued. If a response to treatment does occur, treatment usually should be continued until no further improvement is observed and these laboratory parameters have been stable for about 3 months (see **DOSAGE AND ADMINISTRATION**). It is not known whether continued treatment after that time point is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS INTRON A is contraindicated in patients with a history of hypersensitivity to interferon alfa or any component of the injection.

WARNINGS Moderate to severe adverse experiences may require modification of the patient's dosage regimen, or in some cases, termination of therapy with INTRON A.

Because of the fever and other "flu-like" symptoms associated with INTRON A administration, it should be used cautiously in patients with debilitating medical conditions, such as those with a history of cardiovascular disease (e.g., unstable angina, uncontrolled congestive heart failure), pulmonary disease (e.g., chronic obstructive pulmonary disease), or diabetes mellitus prone to ketoacidosis. Caution should also be observed in patients with coagulation disorders (e.g., thrombophlebitis, pulmonary embolism) or severe myelosuppression.

Patients with platelet counts of less than 50,000/mm^3 should not be administered INTRON A intramuscularly, but instead by subcutaneous administration.

Cardiovascular adverse experiences which include significant hypotension, arrhythmia, or tachycardia of 150 beats per minute or greater, were observed in approximately 3% of the patients studied who had various malignancies and were treated at doses higher than those for hairy cell leukemia. The incidence of these complications in patients with preexisting heart disease is unknown. Hypotension may occur during administration, or up to two days post therapy, and may require supportive therapy including fluid replacement to maintain intravascular volume. Supraventricular arrhythmias occurred rarely and appeared to be correlated with preexisting conditions, and prior therapy with cardiotoxic agents. These adverse experiences were controlled by modifying the dose or discontinuing treatment, but may require specific additional therapy.

Those patients with a recent history of myocardial infarction and/or previous or current arrhythmic disorder, who require INTRON A therapy, should be closely monitored (see **Laboratory Tests**).

Central nervous system effects manifested by depression, confusion and other alterations of mental status were observed in about 2% of hairy cell leukemia patients treated with INTRON A. The overall incidence in a larger patient population with other malignancies treated with higher doses of INTRON A was 10%. More significant obtundation and coma have been observed in some patients, usually elderly, treated at higher doses for other malignant diseases. These effects are usually rapidly reversible. In a few severe episodes, full resolution of symptoms has taken up to three weeks. Patients should be closely monitored until resolution of these effects. Discontinuation of INTRON A Interferon alfa-2b, recombinant for Injection therapy may be required. Narcotics, hypnotics, or sedatives may be used concurrently with caution.

Acute serious hypersensitivity reactions (e.g., urticaria, angioedema, bronchoconstriction, anaphylaxis, etc.) have not been observed in patients receiving INTRON A; however, if such an acute reaction develops, the drug should be discontinued immediately and appropriate medical therapy instituted. Transient cutaneous rashes have occurred in some patients following injection, but have not necessitated treatment interruption.

Laboratory abnormalities which occurred in hairy cell leukemia patients included elevated SGOT and SGPT, which occurred in 4% and 13% of patients, respectively. The overall incidences of laboratory abnormalities in a larger patient population with other malignancies, and treated at higher doses were somewhat higher. These included elevated liver function tests (SGOT, SGPT in 10% of patients) and reductions in granulocyte (20% of patients) and platelet counts (18% of patients) (see **Laboratory Tests**). These abnormalities are usually mild to moderate and transient. Severe abnormalities of these laboratory parameters are usually rapidly reversible upon cessation or reduction of INTRON A therapy.

PRECAUTIONS Information for Patients Patients being treated with INTRON A should be directed in its appropriate use, informed of benefits and risks associated with treatment and referred to the Patient Information Sheet.

Patients should be cautioned not to change brands of Interferon without medical consultation as a change in dosage may result.

It is advised that patients be well hydrated especially during the initial stages of treatment.

Laboratory Tests In addition to those tests normally required for monitoring patients with hairy cell leukemia, the following laboratory tests are recommended for all patients on INTRON A therapy, prior to beginning treatment and then periodically thereafter:

- Standard hematologic tests — including complete blood counts and differential as well as platelet counts.
- Blood chemistries — electrolytes and liver function tests.

Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer, should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis, Impairment of Fertility Studies with INTRON A have not been performed to determine carcinogenicity, or the effect on fertility. Interferon may impair fertility. Mutagenicity studies with INTRON A revealed no adverse effects.

Studies in mice, rats, and monkeys injected with INTRON A for up to one month have revealed no evidence of toxicity. However, due to the known species-specificity of interferon, the effects in animals are unlikely to be predictive of those in man.

Pregnancy Category C Animal reproduction studies have not been conducted with INTRON A. It is also not known whether INTRON A can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. INTRON A should be given to a pregnant woman only if clearly needed. Another interferon alfa preparation has been shown to have abortifacient effects in *Macaca mulatta* (rhesus monkeys) when given at 20 to 500 times the human dose. Therefore, INTRON A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. In studies of interferon use in non-human primates, abnormalities of the menstrual cycle have been observed.

INTRON A Interferon alfa-2b, recombinant for Injection should be used with caution in fertile men and women.

Nursing Mothers It is not known whether this drug is excreted in human milk. However, studies in mice have shown that mouse interferons are excreted into the milk. Because of the potential for serious adverse reactions from INTRON A in nursing infants, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use Safety and effectiveness have not been established in patients below the age of 18 years.

ADVERSE REACTIONS Adverse reactions to INTRON A are dose-related. In studies of patients with malignancies other than hairy cell leukemia, hematologic and hepatic toxicities were more common in patients receiving individual injections greater than 10 million IU, than in patients receiving less than this amount. Hematologic, hepatic, cardiovascular and neurologic toxicities were more common in patients receiving more than 30 million IU per injection than in patients receiving less than that amount.

The following adverse reactions were reported to be possibly or probably related to INTRON A therapy during clinical trials. The most frequently reported adverse reactions were flu-like symptoms, primarily fever, fatigue, and chills which occurred in almost all patients treated. Other organ systems in which reactions occurred in more than 5% of the patients are:

General — Taste alteration, anorexia and weight decrease occurred uncommonly.

Cardiovascular — Mild hypotension was frequently recorded although it was rarely symptomatic. Hypertension also occurred less commonly. Tachycardias occurred uncommonly usually in association with high fevers.

Gastrointestinal System — Nausea was common, although vomiting occurred rarely. Mild diarrhea was reported less commonly.

Hematologic — Mild thrombocytopenia and transient granulocytopenia occurred commonly.

Musculo-Skeletal System — Myalgia and arthralgia occurred commonly, usually in association with other flu-like symptoms.

Nervous System — Headache, somnolence, confusion, and dizziness occurred uncommonly. Ataxia and paresthesia were rare.

Psychiatric — Anxiety, depression and nervousness occurred uncommonly.

Skin and Appendages — Mild pruritus occurred uncommonly. Mild alopecia was uncommon. Various transient, mild skin rashes were seen frequently in patients with hairy cell leukemia.

Other Adverse Reactions — The following adverse reactions were reported with an incidence of 5% or less: leg cramps, constipation, insomnia, herpetic eruptions, non-herpetic cold sores, urticaria, hot flashes, supraventricular arrhythmias, epistaxis, stomatitis, paralytic ileus, dehydration, coagulation disorder (elevated PT and PTT), abnormal vision, tremor, emotional lability, chest pain, pharyngitis and syncope.

Adverse reactions reported rarely (less than 1%) were dyspepsia, purpura, dyspnea, sneezing, oculomotor paralysis, nasal congestion, flatulence, increased saliva, hyperglycemia, and ulcerative stomatitis.

Laboratory Value Changes — Those patient laboratory values which were normal to moderately abnormal (WHO grades 0–2) at baseline that worsened to either severe or life-threatening abnormalities (WHO grades 3 or 4) during some phase of treatment included: WBC, platelets, granulocytes, SGOT, creatinine, SGPT, LDH, and alkaline phosphatase.

DOSAGE AND ADMINISTRATION The recommended dosage of INTRON A for Injection for the treatment of hairy cell leukemia is 2 million IU/m^2 administered intramuscularly (see **WARNINGS**) or subcutaneously 3 times a week. Higher doses are not recommended. The normalization of one or more hematologic variables usually begins within 2 months of initiation of therapy. Improvement in all three hematologic variables may require 6 months or more of therapy.

This dosage regimen should be maintained unless the disease progresses rapidly, or severe intolerance is manifested. If severe adverse reactions develop, the dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. If persistent or recurrent intolerance develops following adequate dosage adjustment, or disease progresses, treatment with INTRON A Interferon alfa-2b, recombinant for Injection should be discontinued. The minimum effective dose of INTRON A has not been established.

At the discretion of the physician, the patient may self-administer the dose. The medication may be administered at bedtime.

HOW SUPPLIED INTRON A Interferon alfa-2b, recombinant for Injection is available in 3-, 5-, 10- and 25-million IU vials, each packaged with its own diluent.

Store INTRON A for Injection, both before and after reconstitution, between 2° and 8°C (36° and 46°F).

JBS-13762805

References: **1.** Data on file, Schering Corporation. **2.** Jacobs AD, Champlin RE, Golde DW: Recombinant α-2-interferon for hairy cell leukemia. *Blood* 1985; 65:1017-1020. **3.** Golomb HM, Jacobs A, Fefer A, et al: Interferon treatment of HCL. *J Clin Oncol*. To be published. **4.** Herberman RB, Ortaldo JR, Rubinstein M, et al: Augmentation of natural and antibody-dependent cell-mediated cytotoxicity by pure human leukocyte interferon. *J Clin Oncol* 1981; 1:149-153. **5.** Schultz RM: Macrophage activation by interferons. *Lymphokine Reports* 1980; 1:63-97. **6.** Herberman RB, Ortaldo JR, Mantovani A, et al: Effect of human recombinant interferon on cytotoxic activity of natural killer (NK) cells and monocytes. *Cell Immunol* 1982; 67:160-167. **7.** Tomida M, Yamamoto Y, Hozumi M: Stimulation by interferon of induction of differentiation of human promyelocytic leukemia cells. *Biochem Biophys Res Comm* 1982; 104(Jan 15):30-37. **8.** Samid D, Chang EH, Friedman RM: Biochemical correlates of phenotypic reversion in interferon-treated mouse cells transformed by a human oncogene. *Biochem Biophys Res Comm* 1984; 119(Feb 29):21-28. **9.** Brouty-Boye D, Wybier-Franqui J, Calvo C, et al: Reversibility of the transformed and neoplastic phenotype. IV. Effects of long-term interferon treatment of C3H/10T½ cells transformed by methylcholanthrene and SV40 virus. *Int J Cancer* 1984; 34:107-112. **10.** Friedman RM: *Interferons: A Primer.* New York, Academic Press, 1981, pp 3-7. **11.** Jonak GJ, Knight E Jr: Selective reduction of c-*myc* mRNA in Daudi cells by human β interferon. *Proc Natl Acad Sci* 1984; 81:1747-1750. **12.** Soslau G, Bogucki AR, Gillespie D, et al: Phosphoproteins altered by antiproliferative doses of human interferon-β in a human bladder carcinoma cell line. *Biochem Biophys Res Comm* 1984; 119:941-948. **13.** Balkwill F, Taylor-Papadimitriou J: Interferon affects both G_1 and $S + G_2$ in cells stimulated from quiescence to growth. *Nature* 1978; 274:798-800. **14.** Salmon SE, Durie BGM, Young L, et al: Effects of cloned human leukocyte interferons in the human tumor stem cell assay. *J Clin Oncol* 1983; 1:217-225. **15.** Welander CE, Morgan TM, Homesley HD, et al: Combined recombinant human interferon alpha$_2$ and cytotoxic agents studied in a clonogenic assay. *Int J Cancer* 1985; 35:721-729.

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* leukemia and related diseases

LEUCOVORIN CALCIUM

LEUCOVORIN CALCIUM INJECTION
Military Depot: NSN 6505-01-054-7008.
50 mg/vial for I.M. use

DESCRIPTION: Each 1 ml ampul of Leucovorin Calcium *Lederle* contains Leucovorin 3 mg as the calcium salt which is the form preferred for intramuscular injection. Preservative: benzyl alcohol 0.9% w/v. The inactive ingredients are sodium chloride 0.56% w/v, Water for Injection qs 100% and sodium hydroxide or hydrochloric acid is used to adjust the pH to approximately 7.7.

Each 50 mg vial of Leucovorin Calcium cryodesiccated powder when reconstituted with 5 ml of sterile diluent contains Leucovorin 10 mg per ml as the calcium salt which is the form preferred for intramuscular injection. Contains no preservative. Dilute only with Bacteriostatic Water for Injection USP which contains benzyl alcohol. The inactive ingredients are sodium chloride 40 mg/vial, and sodium hydroxide or hydrochloric acid qs to pH approximately 8.1. When reconstituted as directed, the resulting solution must be used within seven days. If the product is reconstituted with Water for Injection USP, use immediately.

ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid.

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase. There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg.

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists. (See Warnings.) (b) in the treatment of the megaloblastic anemias due to sprue, nutritional deficiency, pregnancy, and infancy when oral therapy is not feasible.

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient.

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours.

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive.

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

DOSAGE: Megaloblastic anemia: No more than or up to 1 mg daily. There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg; additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

For the treatment of overdosage of folic acid antagonists: To be given in amounts equal to the weight of the antagonist given.

HOW SUPPLIED:
NDC 0205-4004-51—6—1 ml Ampuls
NDC 0205-5330-92—50 mg Vial of Cryodesiccated powder

LEDERLE PARENTERALS, INC.
Carolina, Puerto Rico 00630

Rev. 11/80

LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

Before prescribing Leucovorin Calcium Tablets, please consult complete prescribing information.

INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS: *General:* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother.

Pediatric Use: (See Drug Interactions.)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[9,10,11]

REFERENCES:

1. Nixon PF and Bertino JR: Effective Absorption and Utilization of Oral Formyltetrahydrofolate in Man. *N Engl J Med 286*(4): 175-179, Jan 1972.
2. Ratanasthien K, Blair JA, Leeming RJ, Cooke WT and Melikian V: Folates in Human Serum. *J Clin Pathol 27*: 875-879, 1974.
3. Mehta BM, Gisolfi AL, Hutchison DJ, Nirenberg A, Kellick MG and Rosen G: Serum Distribution of Citrovorum Factor and 5-methyltetrahydrofolate Following Oral and I.M. Administration of Calcium Leucovorin in Normal Adults. *Cancer Treat Rep 62*(3): 345-350, Mar 1978.
4. Data on file, Clinical Research, Lederle Laboratories, Pearl River, NY 10965.
5. Whitehead VM, Pratt R, Viallet A and Cooper BA: Intestinal Conversion of Folinic Acid to 5-methyltetrahydrofolate in Man. *Br J Haematol 22*: 63-72, 1972.
6. Herbert V: Aseptic Addition for *Lactobacillus casei* Assay of Folate Activity in Human Serum. *J Clin Path 19*: 12-16, 1966.
7. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer 41*(1): 36-51, 1978.
8. Frei E, Blum RH, Pitman SW, *et al*: High Dose Methotrexate with Leucovorin Rescue: Rationale and Spectrum of Antitumor Activity. *Am J Med 68*: 370-376, 1980.
9. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulphamethoxazole Inhibition of Haematopoiesis *in Vitro*. *Br J Haematol, 40*(3): 363-367, 1978.
10. Steinberg SE, Campbell CL, Rabinovitch PS, *et al*: The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
11. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX. Toxoplasmosis. *J Infect Dis 135*(3): 493-496, 1977.

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REVIEW

α-Thalassemia in Blacks: Genetic and Clinical Aspects and Interactions With the Sickle Hemoglobin Gene

By Martin H. Steinberg and Stephen H. Embury

Recent advances in molecular genetics have permitted detailed study of the human α-globin genes and the causes of α-thalassemia. In this review, we examine the causes of α-thalassemia in the black population and the consequences of the interactions between α-thalassemia and structural hemoglobin variants.

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THE DETAILED EXPOSITION of the location, structure, and number of α-globin genes using recombinant DNA technology clarified our understanding of the genetic, clinical, and hematologic features of α-thalassemia. In black Americans the heterozygote frequencies of α-thalassemia and HbS are about 0.30 and 0.08, respectively. The hematologic and clinical features of the sickle hemoglobinopathies are influenced by their interactions with α-thalassemia. In this review we discuss α-thalassemia in blacks and its effects upon sickle and other hemoglobinopathies.

GENETICS OF α-THALASSEMIA IN BLACKS

The α-globin gene cluster resides on the short arm of chromosome 16.[1,2] The two α-globin genes are separated by less than 4 kb of DNA[3,4] and code for identical globin chains. The ζ-globin gene[5] is normally expressed only during embryogenesis. A map of the α-globin gene cluster, along with the extent of the deletions that have been described in blacks, is shown in Fig 1. The deletion of a single α-globin gene from one or both chromosomes accounts for most forms of α-thalassemia in this population.[6,7] The prevalence of α-thalassemia varies among the black populations so far examined,[8] but within the United States the heterozygote frequency is about 0.30,[6] while about 2% of this population are homozygotes. By far the most prevalent deletion causing α-thalassemia in blacks removes about 3.7 kb of DNA ($-\alpha^{3.7}$, "rightward deletion").[9] The single α gene associated with this deletion may be an $\alpha_2\alpha_1$ fusion product or the intact α_2 gene.[10] The "leftward" ($-\alpha^{4.2}$) deletion is seen mainly in Asians,[9] but has also been found in blacks.[11,12] The restriction fragments characteristic of both deletions are shown in Table 1.

Chromosomes that have lost both α genes ($--/$) are rare in blacks, and only two instances have been fully characterized (Fig 1). In one patient, the entire α-like gene cluster was missing.[13] The second patient had a deletion of at least 27 kb of DNA, but in distinction to the first, the functional ζ-globin gene was present.[14] Rarely, α-thalassemia may be a result of a nondeletion, or point mutation.[15]

CLINICAL AND HEMATOLOGIC FEATURES OF α-THALASSEMIA IN BLACKS

α-Thalassemias in blacks are rarely clinically significant. The major virtue in detection is to distinguish them: (*a*) from iron deficiency, avoiding unwarranted treatment or diagnostic testing; and (*b*) from β thalassemia, so that misinformed genetic counseling is not provided.

Individuals with the $-\alpha/\alpha\alpha$ genotype are not anemic, and usually have a normal mean corpuscular volume (MCV). The $-\alpha/-\alpha$ genotype is associated with microcytic erythrocytes and sometimes with mild anemia.

Because the $--/$ haplotype is so rare in blacks,[6,13,14] the clinically important forms of α-thalassemia that exist in other racial groups are either seen rarely (HbH disease) or have not yet been described (hydrops fetalis). The proportion of HbH in HbH disease may vary from <1% to >20%, and the lower concentrations often seen in black patients with this condition[16] may escape detection.

DIAGNOSIS

The detection of α-thalassemia in blacks is difficult because of the mild hematologic changes that are present. There are no characteristic alterations in hemoglobin composition which readily permit detection of the simple heterozygote. The presence of mild microcytosis, in the absence of

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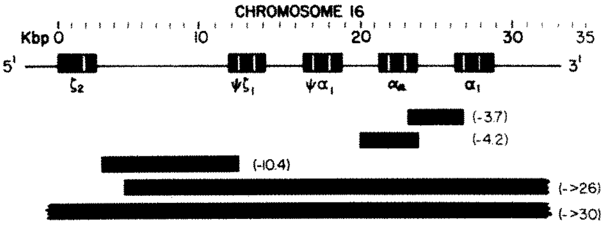

Fig 1. Map of the α-globin-like gene cluster showing the location of the functional ζ- and α-globin genes and their accompanying pseudogenes. Beneath this map are the various deletions described in blacks and accounting for different forms of α-thalassemia. The deletion between the ζ-genes occurs in about 2% to 3% of blacks and is not associated with a thalassemia phenotype (data from Rappaport et al[5a] and Felice[5b]).

iron deficiency or heterozygous β-thalassemia, suggests the presence of α-thalassemia. In addition, α-thalassemia reduces the proportion of HbS in carriers of sickle cell trait (see below).

Three approaches have been used to diagnose α-thalassemia. The most reliable is restriction mapping of the α genes. The relative rates of α- and β-globin synthesis can also be measured. In neonates α-thalassemia may be detected by finding increased levels of Hb Barts ($\gamma4$) in cord blood. Restriction mapping is rarely indicated clinically. The considerable overlap of globin synthesis ratios of $-\alpha/\alpha\alpha$ individuals with those of subjects having either $-\alpha/-\alpha$ or $\alpha\alpha/\alpha\alpha$ blunts the usefulness of this method. Likewise, cord blood screening for Hb Barts levels cannot reliably distinguish $-\alpha/\alpha\alpha$ from $-\alpha/-\alpha$ and may not always detect $-\alpha^{3.7}$ heterozygotes.

α-THALASSEMIA AND HbAS

HbAS (sickle cell trait) is generally a benign condition, although certain uncommon complications are known to occur.[17] The interaction of α-thalassemia with HbAS results in easily detectable hematologic changes,[18] and the effects of the various α-thalassemia genotypes are shown in Table 2.[19-22] Most notable is the influence of different α-globin genotypes on the percentage of HbS.

Table 1. α-Globin Gene Deletions in Blacks

	Characteristic Restriction Pattern*	
Genotype	α-gene probe	ζ-gene probe
$-\alpha^{3.7}\alpha/\alpha\alpha$	Bam HI 14,10	11,6
	Bgl II 16.0,12.5,7.0	16.0,12.5,11.5,10.5
$-\alpha^{4.2}\alpha/\alpha\alpha$	Bam HI 14,10	11,6
	Bgl II 12.5,7.0	12.5,11.5,10.5,8
$-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$	Bam HI 10	11,6
	Bgl II 16	16,11.5
$-\alpha^{3.7}\alpha/-\alpha^{4.2}\alpha$	Bam HI 10	11,6
	Bgl II 16,7	16,11.5,8.5
$--/-\alpha^{3.7}\alpha$	Bam HI 10	11,6
	Bgl II 16	16,11.5,8.5
$\alpha\alpha/\alpha\alpha$	Bam HI 14	11,6
	Bgl 12.5,7.0	12.5,11.5

*The α-gene probe detects only fragments of DNA that contain α-gene sequences while the ζ-probe detects only DNA that contains ζ-sequences. The size in kilobases for each restriction fragment is given next to the enzyme used to cleave DNA.[4,6,7,13,14]

Table 2. The Hematology of α-Thalassemia and Sickle Cell Trait

α-Globin Genotype	Hb (g/dL)	MCV (fL)	α/β*	HbS (%)
$\alpha\alpha\alpha/\alpha\alpha$	13-15	80-90	—	40†
$\alpha\alpha/\alpha\alpha$	13-15	80-90	1.0	35-45
$-\alpha/\alpha\alpha\alpha$	13-15	80-90	—	35-45
$-\alpha/\alpha\alpha$	13-14	75-85	0.85	30-35
$-\alpha/-\alpha$	12-13	70-75	0.75	25-30
$--/-\alpha$	7-10	50-55	0.50	17-25

*Ratio of radioactivity incorporated into α- and β-globin chains. The normal value is about 1 with ratios of less than 0.85 indicative of deficient α-globin synthesis.

†HbS levels in this genotype, studied by Higgs et al,[22] were higher than that in $\alpha\alpha/\alpha\alpha$ individuals. The ranges or values given are averages taken from the literature and may vary significantly among individuals.[19-22] About 50 patients with the $\alpha\alpha/\alpha\alpha$, 40 patients with $-\alpha/\alpha\alpha$, and 20 patients with the $-\alpha/-\alpha$ genotype have been reported. Far fewer patients with the other α-globin genotypes have been examined.

Although most data indicate that HbAS has no significant effect on clinical morbidity or mortality,[17,23] certain studies suggest that the fraction of HbS may influence hematologic values and clinical events. In a study of 355 black men with HbAS who were stratified on the basis of their HbS percentage, this fraction correlated directly with MCV and packed cell volume (PCV) but not with the incidence of thrombotic events.[24] However, hematuria was noticed in only three subjects with HbS <35% compared to nine patients with >35% HbS.[24] This observation is consistent with the recent finding that subjects with lower fractions of HbS have greater urinary concentrating ability.[25] Moreover, it suggests that the fractional HbS content of sickle trait erythrocytes may influence the severity of certain clinical complications.

Other reports have suggested that besides the fractional HbS content, there may be other modifying influences on events that punctuate the clinical course of sickle cell trait. Particularly intriguing are the findings of racial differences in the prevalence of splenic infarction in patients at high altitude.[26,27] In view of the profound influence of intraerythrocytic deoxy-HbS concentration on polymerization kinetics,[28] it is tempting to speculate that the increased risk of nonblack individuals may relate to known differences affecting red cell hydration.[29,30] Compared to caucasian controls, erythrocytes from black individuals have lower Na-K ATPase activity and greater Na, K, and total cation content, findings that are likely to result in greater cell water content and lower mean cellular Hb concentrations (MCHC). While not influencing the fractional HbS content of red cells, these changes are predicted to retard HbS polymerization by reducing its intracellular concentration in black individuals.

α-THALASSEMIA AND OTHER HEMOGLOBIN VARIANTS

When present with positively charged β-chain variants, like HbC[31] or HbE,[32] α-thalassemia reduces the proportion of the abnormal hemoglobin. This reduction is more striking in the case of HbE, probably as a result of the intrinsically thalassemic behavior of the β^E-gene.[33-35] In the case of negatively charged β-variants, like HbJ Baltimore and HbN, the presence of α-thalassemia enhances the assembly of the

HbJ or HbN tetramer and increases the level of the variant hemoglobin.[36]

HbG Philadelphia (α^{68}Asn → Lys) is the most common α-globin variant in blacks. The proportion of HbG is dependent upon the number of functional α genes,[37,38] and most studies indicate that HbG levels of about 30% are associated with the $-\alpha^G/\alpha\alpha$ genotype while levels near 50% are associated with the $-\alpha/-\alpha^G$ gene arrangement. Patients with 100% HbG have $-\alpha^G/-\alpha^G$, while those with HbG and HbH disease have the $--/-\alpha^G$ genotype.[39] Patients with 20% to 25% HbG appear to have no deleted α genes ($\alpha\alpha/\alpha\alpha^G$).[40] HbG has also been reported in association with HbAS-α-thalassemia and HbSS-α-thalassemia.[38,41]

α-THALASSEMIA AND SICKLE CELL ANEMIA

One situation in which the clinical impact of α thalassemia has become better understood in recent years is its coexistence with sickle cell anemia (HbSS).[42] The availability of gene mapping for determining α-globin genotypes has provided objective parameters with which to compare the more subjective clinical variables of sickle cell disease,[43] and has served as a useful tool for dissecting various pathophysiological determinants.[44] These studies have also permitted reinterpretation of the earlier reports describing the effects of α-thalassemia on HbSS that were based on less objective diagnostic techniques.[45-53]

The first report employing gene mapping documented that HbSS individuals who were α-thalassemic, due to the deletion of either one or two α-globin genes, were less anemic than those who were nonthalassemic.[54] It was also found that with decreasing α-globin gene number there was a commensurate reduction in the mean corpuscular hemoglobin (MCH) and MCV, and in the estimated MCHC of sickle erythrocytes. It was presumed that the profound effect of MCHC on HbS polymerization[28,55] was responsible for the milder degree of anemia and was speculated that the greater benefits of single gene deletion *vis-a-vis* double deletion may have been related either to detrimental effects associated with greater imbalance of globin synthesis or to the small numbers of $-\alpha/-\alpha$ subjects in the study. Since this report, the epistatic influences of α-thalassemia on degree of anemia[56,57] and MCHC[56,58,59] have been confirmed, and improved circulatory survival of sickle cells from α-thalassemic individuals demonstrated that their milder anemia is due to less rapid hemolysis.[60] The hematologic effects of α-thalassemia upon HbSS are shown in Table 3.

Several published studies of this interaction have ignored the influence of $-\alpha/\alpha\alpha$ and compared only the $-\alpha/-\alpha$ with the normal $\alpha\alpha/\alpha\alpha$ genotype. This was due either to the notion that a single gene deletion has minimal effect on the pathophysiology of sickle cell disease[56] or to using diagnostic techniques that are unable to reliably detect the $-\alpha/\alpha\alpha$ genotype. This approach limits the number of α-thalassemic HbSS subjects available for study, and in $-\alpha/-\alpha$ individuals, may result in the beneficial effects of α-thalassemia being masked by potential negative influences of more imbalanced globin synthesis.

Table 3. The Hematology of α-Thalassemia and Sickle Cell Anemia*

α-Globin Genotype	Hb (g/dL)	MCV (fL)	Reticulocytes	HbA$_2$ (%)
$\alpha\alpha/\alpha\alpha$	8.0 ± 1.1	92 ± 7	11 ± 6	2.8 ± 0.4
$\alpha\alpha\alpha/\alpha\alpha$				
$-\alpha/\alpha\alpha$	8.6 ± 1.1	83 ± 7	9 ± 6	3.3 ± 0.6
$-\alpha/-\alpha$	9.2 ± 1.3	72 ± 4	7 ± 5	3.8 ± 0.4

*These average values (± 1 SD) are taken from studies that employed α-globin gene mapping.[54,56,57] There were 61 patients studied in the $-\alpha/-\alpha$, 101 in the $-\alpha/\alpha\alpha$ group, and 186 in the $\alpha\alpha/\alpha\alpha$ group.

Studies of the effect of α-thalassemia on clinical features besides anemia have produced conflicting results. One study that employed gene mapping reported less frequent leg ulcers and acute chest syndromes in subjects with α-thalassemia,[56] while another gene mapping study was unable to confirm this effect.[57] This study segregated patients by MCV and MCH and globin biosynthesis studies, as well as gene mapping, and separate analyses were done on each group. While the gene-mapped group was the smallest, the results in each group were similar and suggested that neither reduced MCV and MCH, nor α-thalassemia were accompanied by a reduction in vaso-occlusive complications. Atlhough the analyses of patients categorized as α-thalassemic by globin synthesis ratios could not address the issue of single *v* double gene deletion, the finding of increased osteonecrosis among α-thalassemic HbSS subjects is consistent with an earlier report.[61] Another report suggests that retinopathy occurs more frequently in subjects with the hematologic characteristics of coexistent α-thalassemia and HbSS.[62] These paradoxically adverse vaso-occlusive effects associated with α-thalassemia have been attributed[63] to the detrimental rheologic effects caused by higher hematocrits.[64] One group has reported that the presence of α-thalassemia may be associated with increased life expectancy,[58,65] an effect that

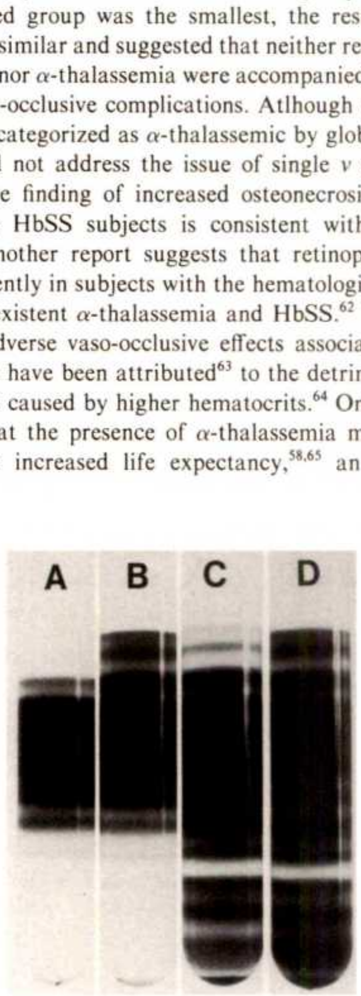

Fig 2. Effect of α-globin gene number on the density of sickle cells. Twelve-step analytic Stractan gradients consisting of 1-mL fractions spanning a density range of 1.120 g/mL to 1.070 g/mL in equal increments. Prior to centrifugation, 125 μL of packed erythrocytes from (A) a normal HbAA control, (B) an HbSS subject having two α-globin genes, (C) an HbSS subject having three α-globin genes, and (D) a nonthalassemic HbSS subject having four α-globin genes. Reprinted with permission.[66]

implies an important role for the degree of anemia in determining survival.

The consistent reports of less severe anemia have stimulated more detailed studies of the mechanisms by which α-thalassemia affects sickle cell anemia. In this regard, density gradient studies demonstrated that α-thalassemic individuals had fewer dehydrated dense cells (Fig 2) and confirmed[56] the interrelated finding of fewer irreversibly sickled cells.[66] Using a laser diffraction viscosimeter to measure the deformability of sickle cells revealed that cells from individuals with α-thalassemia had better deformability.[66] When cell fractions having the same density were studied, their deformability was the same regardless of α-globin gene numbers. It was concluded that the beneficial effect of α-thalassemia upon the deformability of sickle cells was due to fewer dense cells, and that the basic mechanism of the effect was related to improved cell hydration and lower MCHC.

The profound effect of MCHC on both the kinetics[28] and the extent[55] of intra-erythrocytic polymerization made this hypothesis an attractive one. However, the large fluctuations in cation and water content characteristic of sickle cells provided an element of uncertainty about whether improved cell hydration was the basic mechanism by which α-thalassemia benefited sickle cells. The possibility remained that the improved hydration of these cells was secondary to some more fundamental property that was the proximate cause of the beneficial effect. In this regard, the discovery that α-thalassemia protects against the abnormally large cation changes (Figure 3)[67] that eventually lead to severe cellular dehydration[68] was of interest. Taken together with the finding that sickling-induced membrane stretching is the direct cause of cellular potassium loss during deoxygenation,[69] these data suggested that membrane surface-area-to-volume relationships may be important. Indeed, preliminary data demonstrated that sickle cells from α-thalassemic individuals had lower cell volumes and hemoglobin contents and relatively greater surface areas compared to nonthalassemic sickle cells.[67,70] These features convey to such cells the property of increased membrane redundancy, which provides a larger reserve of internal volume per given amount of polymer. Thus, they are relatively protected against the deleterious consequences of membrane stretching during deoxygenation.

In summary, it would appear that the fundamental mechanism by which α-thalassemia benefits sickle cells is related to greater membrane redundancy and the protection it provides against sickling-induced membrane stretching, cation loss, and cell dehydration. In turn, the lower MCHC of these better-hydrated cells retards the polymerization of deoxyhemoglobin S. This construction may also explain the recent finding that α-thalassemia does not affect the degree of anemia in young children.[71,72] That is, their persistent splenic function may result in conditioning of red cell membranes,[73] thereby depriving children with sickle cell disease and α-thalassemia of the beneficial effects of membrane redundancy. This scenario, however, does not explain the greater duration of splenic function[74] and the prolonged period at risk for splenic sequestration[75] reported for children with coexistent HbSS and α-thalassemia. These effects which antedate the loss of splenic function suggest that there may be some beneficial effect of α-thalassemia unrelated to membrane redundancy, perhaps a slight primary improvement in cell hydration. Alternatively, it is conceivable that very subtle improvements in sickle cells are sufficient to preserve splenic tissue while the greater benefits associated with enhanced membrane redundancy are required to affect the degree of anemia.

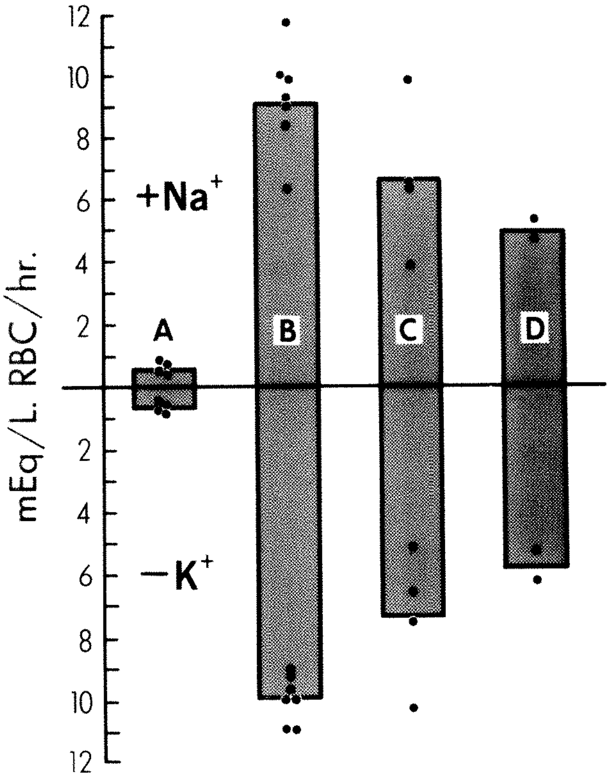

Fig 3. Changes in net sodium and potassium content of red cells during deoxygenation. Net sodium influx ($+Na^+$) is shown above midline and net potassium efflux ($-K^+$) below. The ordinate scale shows net cation changes. (A) Data for cells from four normal control subjects without sickle cell anemia. (B) Data for cells from seven HbSS subjects having four α-globin genes. (C) Data for cells from four HbSS subjects having three α-globin genes. (D) Data for cells from two subjects having two α-globin genes. For each group, panels representing mean net cation changes and all data points are shown. Reprinted with permission.[67]

The ability to subdivide HbSS patients according to α-globin genotypes has provided an unprecedented opportunity to dissect some aspects of the pathophysiology of sickle cell disease. The consideration of α-globin gene number is necessary for complete understanding of many of the pathophysiologic processes. At this time, the important issues raised by these analyses are only beginning to be answered.

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REFERENCES

1. Deisseroth A, Nienhuis A, Turner P, Velez R, Anderson WF, Ruddle F, Lawrence J, Creagan R, Kucherlapati R. Localization of the human α-globin structural gene to chromosome 16 in somatic cell hybrids by molecular hybridization. Cell 12:205, 1977

2. Koeffler HP, Sparkes RS, Stang H, Mohandas T. Regional assignment of genes for human α-globin and phosphoglycolate phosphatase to the short arm of chromosome 16. Proc Natl Acad Sci USA 78.7015, 1981

3. Orkin SH. The duplicated human α-globin genes lie close together in cellular DNA. Proc Natl Acad Sci USA 75:5950, 1978

4. Embury SH, Dozy AM, Lebo RV, Kan YW: Organization of the α-globin genes in the Chinese α-thalassemia syndromes. J Clin Invest 63:1307, 1979

5. Proudfoot NJ, Gill A, Maniatis T· The structure of the human zeta-globin gene and a closely linked nearly identical pseudo-gene. Cell 3:553, 1982

5a. Rappaport EF, Schwartz E, Poncz M, Surrey S: Biochem Biophys Res Commun 125.817, 1984

5b. Felice AE. Human Genetics (in press)

6. Dozy AM, Kan YW, Embury SH, Mentzer WC, Wang WC, Lubin B, Davis JR, Koenig HM: α-Globin gene organization in blacks precludes the severe form of α-thalassaemia. Nature 280.605, 1979

7. Higgs DR, Old JM, Clegg JB, Pressley L, Hunt DM, Weatherall DJ, Serjeant GR: Negro α-thalassaemia is caused by deletion of a single α-globin gene. Lancet II:272, 1979

8. Nagel RL, Fabry ME, Paginer J, Zohoun I, Wajcman H, Baudin V, Labie D: Hematologically and genetically distinct forms of sickle cell anemia in Africa: The Senegal type and the Benin type N Engl J Med 312:880, 1985

9. Embury SH, Miller JA, Dozy AM, Kan YW, Chan V, Todd D: Two different molecular organizations account for the single α-globin gene of the α-thalassemia-2 genotype. J Clin Invest 66·1319, 1980

10. Higgs DR, Hill AVS, Bowden DK, Weatherall DJ, Clegg JB: Independent recombination events between the duplicated human α-globin genes Implications for their concerted evolution. Nucleic Acids Res 12:6965, 1984

11. Embury SH, Gholson MA, Gillette P, Rieder RF, and National Cooperative Study of Sickle Cell Disease: The leftward deletion α-thal-2 haplotype in a black subject with hemoglobin SS. Blood 65 769, 1985

12 Wong SC, Chang LS, Olivieri NF, Poon AO, Ali MAM, Graves DJ: Heterozygosity for two genotypes of α-thalassemia-2 Hematological, biosynthetic and DNA studies. Hemoglobin 9.111, 1985

13. Felice AE, Cleek MP, McKie K, McKie V, Huisman THJ. The rare α-thalassemia-1 of blacks is a $\zeta\alpha$-thalassemia-1 associated with deletion of all α- and ζ-globin genes. Blood 63:1253, 1984

14 Steinberg MH, Coleman MB, Adams JG, Hartmann RC, Saba H, Anagnou NP: A new gene deletion in the α-like globin gene cluster as the molecular basis for the rare α-thalassemia-1 $(--/\alpha\alpha)$ in blacks HbH disease and sickle cell trait. Blood 67·469, 1986

15 Steinberg MH, Coleman MB, Adams JG: Non-deletion alpha-thalassemia in blacks. Clin Res 34:217a, 1986

16 Schwartz E, Atwater J: α-Thalassemia in the American Negro J Clin Invest 51:412, 1972

17 Heller P, Best WR, Nelson RB, Becktel J: Clinical implications of sickle cell trait and glucose-6-phosphate dehydrogenase deficiency in hospitalized black male patients. N Engl J Med 300:1001, 1979

18. Steinberg MH, Adams JG, Dreiling BJ: Alpha-thalassaemia in adults with sickle cell trait. Br J Haematol 30:31, 1975

19. Embury SH, Dozy AM: The α-globin genotype as a determinant of hematologic parameters in sickle cell trait, in Sigler PH (ed): The Molecular Basis of Mutant Hemoglobin Dysfunction, New York, North Holland Elsevier, 1981, p 63

20. Higgs DR, Pressley L, Serjeant GR, Clegg JB, Weatherall DJ: The genetics and molecular basis of alpha thalassaemia in association with HbS in Jamaican negroes. Br J Haematol 47.43, 1981

21. Higgs DR, Weatherall DJ. Alpha thalassemia. Curr Top Hematol 4:37, 1983

22. Higgs DR, Clegg JB, Weatherall DJ, Serjeant BE, Serjeant GR: The interaction of the $\alpha\alpha\alpha$ globin gene haplotype and sickle haemoglobin. Br J Haematol 57:671, 1984

23. Sears DA. The morbidity of sickle cell trait: A review of the literature. Am J Med 64:1021, 1978

24. Kennedy A, Walsh DA, Nicholson R, Adams JG III, Steinberg MH· Influence of HbS levels upon the hematological and clinical characteristics of sickle cell trait. Am J Hematol 22:51, 1986

25. Reeves JD, Lubin BH, Embury SH: Renal complications of sickle cell trait are related to the percent of HbS. Blood 64·52a, 1984 (suppl 1)

26. Lane PA, Githens JH: Splenic syndrome at mountain altitudes in sickle cell trait: Its occurrence in non-black persons. JAMA 253:2251, 1985

27. Castro O, Finch SC: Splenic infarction in sickle-cell trait: Are whites more susceptible? N Engl J Med 291 630, 1974

28 Eaton WA, Hofrichter J, Ross RD: Delay time of gelation: A possible determinant of clinical severity in sickle cell disease. Blood 47:621, 1976

29. Muno-Faure AD, Hill DM, Anderson J: Ethnic differences in human red blood sodium concentration. Nature 231:457, 1971

30. Lasker N, Hopp L, Grossman S, Bamforth R, Aviv A: Race and sex differences in erythrocyte Na^+, K^+, and Na^+-K^+-adenosine triphosphatase. J Clin Invest 75.1813, 1985

31 Steinberg MH: Haemoglobin C/α-thalassaemia: Haematological and biosynthetic studies. Br J Haematol 30:337, 1975

32. Wasi P, Na-Nakorn S, Pootrakul P. The α-thalassaemias. Clin Haematol 3:383, 1974

33 Traeger J, Wood WG, Clegg JB, Weatherall DJ, Wasi P. Defective synthesis of HbE is due to reduced levels of β^E mRNA. Nature 288·497, 1980

34. Benz EJ Jr, Berman BW, Tonkonow BL, Coupal E, Coates T, Boxer LA, Altman A, Adams JG. Molecular analysis of the β-thalassemia phenotype associated with inheritance of hemoglobin E ($\alpha_2\beta^{26}$glu → lys). J Clin Invest 68:118, 1981

35. Orkin SH, Kazazian HH, Antonarakis SE, Ostrer H, Goff SC, Sexton JP: Abnormal RNA processing due to the exon mutation of the β^E-globin gene. Nature 300:768, 1982

36. Bunn HF, McDonald MJ: Electrostatic interactions in the assembly of haemoglobin. Nature 306:498, 1983

37. Surrey S, Okene-Frempong K, Rappaport E, Atwater J, Schwartz E. Linkage of $\alpha^{\text{G-Philadelphia}}$ to α-thalassemia in African-Americans. Proc Natl Acad Sci USA 77 4885, 1980

38 Felice AE, Oxdonmey R, Headlee ME, Huisman THJ. Organization of α-chain genes among HbG-Philadelphia heterozygotes in association with HbS, β-thalassemia, and α-thalassemia-2. Biochem Genet 20.689, 1982

39. Milner PF, Huisman THJ· Studies on the proportion and synthesis of haemoglobin G Philadelphia in red cells of heterozy-

gotes, a homozygote and a heterozygote for both haemoglobin G and α-thalassaemia. Br J Haematol 34:207, 1976

40 Bruzdzinski CJ, Sisco KL, Ferrucci SJ, Rucknagel DL: The occurrence of the α^G-Philadelphia globin allele on a double locus chromosome. Am J Hum Genet 36:101, 1984

41. Charache S, Zinkham WH, Dickerson JD, Brimhall B, Dover GJ: Hemoglobin SC, SS/G Philadelphia and SO Arab diseases. Diagnostic importance of an integrative analysis of clinical hematologic and electrophoretic findings. Am J Med 62:439, 1977

42. Embury SH· The interaction of coexistent α thalassemia and sickle cell anemia A model for the clinical and cellular results of diminished polymerization? Ann NY Acad Sci 445:37, 1985

43. Steinberg MH, Hebbel RP: The clinical diversity of sickle cell anemia: Genetic and cellular modulation of disease severity. Am J Hematol 14:405, 1983

44. Embury SH: The clinical pathophysiology of sickle cell disease. Annu Rev Med 37:361, 1986

45. Weatherall DJ, Clegg JB, Blankson J, McNeil JR: A new sickling disorder resulting from interaction of the genes for haemoglobin S and α-thalassaemia. Br J Haematol 17·517, 1969

46. Van Enk A, Lang A, White JM, Lehmann H: Benign obstetric history in women with sickle-cell anaemia associated with α-thalassaemia. Br Med J 4:524, 1972

47. Kim HC, Weierbach RG, Friedman S, Schwartz E: Detection of sickle α- or β^0-thalassemia by studies of globin biosynthesis. Blood 49:785, 1977

48. Honig GR, Koshy M, Mason G, Vida LN: Sickle cell syndromes. II. The sickle cell anemia-α-thalassemia syndrome. J Pediatr 92:556, 1978

49. Natta C: Failure of the α-thalassemia gene to decrease the severity of sickle cell anemia Blood 51:1163, 1978

50. Huisman THJ: Sickle cell anemia as a syndrome: A review of diagnostic features. Am J Hematol 61:173, 1979

51. Felice AE, Webber B, Miller A, Mayson SM, Harris HF, Henson JB, Gravely ME, Huisman THJ. The association of sickle cell anemia with heterozygous and homozygous α-thalassemia-2. In vitro Hb chain synthesis. Am J Hematol 6:91, 1979

52. Powars DR, Schroeder WA, Weiss JN, Chan LS, Azen SP: Lack of influence of fetal hemoglobin levels or erythrocyte indices on the clinical severity of sickle cell anemia. J Clin Invest 65:732, 1980

53. Altay C, Gravely ME, Joseph BR, Williams DF: Alpha-thalassemia-2 and the variability of hematological values in children with sickle cell anemia. Pediatr Res 15:1093, 1981

54. Embury SH, Dozy AM, Miller J, Davis JR Jr, Kleman KM, Preisler H, Vichinsky E, Lande WN, Lubin BH, Kan YW, Mentzer WC: Concurrent sickle-cell anemia and α-thalassemia. Effect on severity of anemia. N Engl J Med 306:270, 1982

55. Noguchi CT, Schechter AN: The intracellular polymerization of sickle hemoglobin and its relevance to sickle cell disease. Blood 58:1057, 1981

56. Higgs DR, Aldridge BE, Lamb J, Clegg JB, Weatherall DJ, Hayes RJ, Grandison Y, Lowrie Y, Mason KP, Serjeant BE, Serjeant GR: The interaction of alpha-thalassemia and homozygous sickle cell disease. N Engl J Med 306:1441, 1982

57. Steinberg MH, Rosenstock W, Coleman MB, Adams JG, Platica O, Cedeno M, Rieder R, Wilson JT, Milner P, West S, and the Cooperative of Sickle Cell Disease: Effects of thalassemia and microcytosis upon the hematological and vaso-occlusive severity of sickle cell anemia. Blood 63:1353, 1984

58. Fabry ME, Mears JG, Patel P, Shaefer-Rego K, Carmichael LD, Martinez G, Nagel RL: Dense cells in sickle cell anemia: The effects of gene interaction. Blood 64:1042, 1984

59. Noguchi CT, Dover GJ, Rodgers GP, Serjeant GR, Antonarakis SE, Anagnou NP, Higgs DR, Weatherall DJ, Schechter AN: Alpha thalassemia changes erythrocyte heterogeneity in sickle cell disease J Clin Invest 75:1632, 1985

60 de Ceulaer D, Higgs DR, Weatherall DJ, Hayes RJ, Serjeant BE, Serjeant GR· α-Thalassemia reduces the hemolytic rate in homozygous sickle cell disease. N Engl J Med 309:189, 1983

61. Hawker H, Neilson H, Hayes RJ, Serjeant GR: Haematological factors associated with avascular necrosis of the femoral head in homozygous sickle cell disease. Br J Haematol 50:29, 1982

62. Hayes RJ, Condon PI, Serjeant GR: Haematological factors associated with proliferative retinopathy in homozygous sickle cell disease. Br J Ophthalmol 65:29, 1981

63. Serjeant BE, Mason KP, Kenny MW, Stewart J, Higgs DR, Weatherall DJ, Hayes RJ, Serjeant GR: Effect of alpha thalassaemia on the rheology of homozygous sickle cell disease. Br J Haematol 55:479, 1983

64. Chien S: Rheology of sickle cells and erythrocyte content. Blood Cells 3:283, 1977

65. Mears JG, Lachman H, Labie D, Nagel RL: Alpha-thalassemia is related to prolonged survival in sickle cell anemia. Blood 62:286, 1983

66 Embury SH, Clark MR, Monroy G, Mohandas N. Concurrent sickle-cell anemia and α-thalassemia: Effect on pathological properties of sickle erythrocytes. J Clin Invest 73:116, 1984

67. Embury SH, Backer K, Glader BE: Monovalent cation changes in sickle erythrocytes: A direct reflection of α-globin gene number. J Lab Clin Med 106.75, 1985

68. Fabry ME, Nagel RL: The effect of deoxygenation on red cell density: Significance for the pathophysiology of sickle cell anemia. Blood 60:1370, 1982

69. Mohandas N, Rossi ME, Clark MR: Association between morphologic distortion of sickle cells and deoxygenation-induced cation permeability increase. Blood 68:450, 1986

70 Embury SH, Oliver M, Kropp J: The beneficial effect of α thalassemia on sickle cell anemia (SCA) is related to increased membrane redundancy. Blood 66:58a, 1985 (suppl 1)

71. Felice AE, McKie VC, McKie K, Huisman THJ: Deletions and duplications of α or ζ globin genes in children with sickle cell anemia. Blood 64:47a, 1984 (suppl 1)

72. Stevens MCG, Maude GH, Beckford M, Gradison Y, Mason K, Taylor B, Serjeant BE, Higgs DR, Teal H, Weatherall DJ, Serjeant GR: Alpha thalassemia and the hematology of homozygous sickle cell disease in childhood. Blood 67.411, 1986

73. Lux SE, John KM: Isolation and partial characterization of a high molecular weight red cell membrane protein complex normally removed by the spleen. Blood 50:625, 1977

74 Serjeant GR, Foster K, Serjeant BE: Red cell size and the clinical and haematological features of homozygous sickle cell disease. Br J Haematol 48:445, 1981

75 Mears JG, Schoenbrum M, Schaefer KE, Bestak M, Radel E: Frequent association of alpha thalassemia with splenic sequestration crisis and splenomegaly in sickle cell (SS) subjects. Blood 60:47a, 1982 (suppl 1)

Lymphokine(s) From Isolated T Lymphocyte Subpopulations Support Multilineage Hematopoietic Colony and Megakaryocytic Colony Formation

By L. Kanz, G.W. Löhr, and A.A. Fauser

Conditioned medium derived from peripheral mononuclear low-density cells stimulated with phytohemagglutinin (PHA) supports the growth of noncommitted hematopoietic progenitors from marrow and peripheral blood cells. These immature progenitors (CFU-GEMM) can be identified in culture as multilineage hematopoietic colonies containing erythroblasts, eosinophilic, basophilic and neutrophilic granulocytes, megakaryocytes, macrophages, and T and B lymphocytes. In this report, we describe the effect of lymphokines released from purified T lymphocyte preparations of helper (T_4) and suppressor/cytotoxic (T_8) phenotype derived from peripheral blood on the growth of multilineage hematopoietic colonies and megakaryocytic colonies. It was found that PHA-stimulated lymphocytes of T_4 phenotype and, to a lesser extent, of T_8 phenotype elaborate lymphokine(s) that support the growth and development of multilineage colonies (CFU-GEMM), granulopoietic colonies (CFU-C), erythroid bursts (BFU-E) and megakaryocytic colonies (CFU-M) by nonadherent and T cell-depleted bone marrow cells.

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SUPERNATANTS from an activated mouse inducer T cell clone have been identified which stimulate the proliferation of murine granulopoietic colonies, and T and B lymphocytes as well as mast cells.[1] cDNA derived from these T cells was shown to encode a hematopoietic growth factor, presumed to be interleukin 3, with stimulatory activites for noncommitted and committed murine hemopoietic progenitors.[2]

Factors with diverse biological activities are also released from phytohemagglutinin (PHA)-stimulated human peripheral mononuclear cells. These factors support human multilineage colony formation (CFU-GEMM), erythroid burst (BFU-E), granulopoietic colony (CFU-C), and megakaryocytic colony (CFU-M) formation.[3-8] Recently, it has been demonstrated that a T cell-conditioned medium stimulated in the presence of PHA promotes the growth of human pluripotent stem cells.[9] Because those cultures had not been depleted of T cells, colony growth may have been the result of a direct interaction of progenitor cells with T cell-conditioned medium or the result of the induction of endogenous colony-stimulating factor(s) by activated T cells

We studied conditioned media derived from T lymphocyte subpopulations that were highly purified with the use of a fluorescence-activated cell sorter (FACS); we evaluated the capacity of T_4 and T_8 cell-conditioned medium (PHA-T_4CM and PHA-T_8CM) to stimulate colony growth of nonadherent and T cell depleted human bone marrow cells with particular emphasis on its capacity to promote multilineage colony and megakaryocytic colony formation.

MATERIALS AND METHODS

Bone marrow specimens. Bone marrow samples were obtained from volunteers after written informed consent, with the approval of the Ethical Review Committee at the University of Freiburg. The specimens were obtained from the posterior iliac crest. Peripheral blood cells which were used to prepare conditioned media were also obtained from healthy volunteers.

Preparation of leukocyte-conditioned medium (PHA-LCM). Mononuclear cells of density <1.077 g/mL were obtained by centrifugation in Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden); 1 × 10^6 cells/mL were incubated with 1% human serum albumin (HSA, Sigma, Munich, FRG) and 1% PHA (Wellcome HA15, Research Triangle Park, NC) in Iscove's modified Dulbecco's medium (IMDM, GIBCO Laboratories, Grand Island, NY) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The supernatant was harvested after 4 days of culture

Preparation of PHA-T_4CM and PHA-T_8CM Mononuclear cells (1 × 10^7) of density <1.077 g/mL from peripheral blood were incubated with the monoclonal antibody OKT4 or OKT8 (ORTHO Diagnostic Systems, Heidelberg, FRG) for 15 minutes at 4 °C, washed with IMDM, and subsequently labeled with FITC-conjugated $F(ab')_2$ fragments of goat-anti-mouse IgG (Jackson Immuno Research, Hamburg, FRG) for 15 minutes. Control cells were labeled with mouse IgG (Coulter Electronics, Krefeld, FRG) and the second antibody. For simultaneous isolation of T_4 and T_8 positive cells, double labeling with anti-T_4-phycoerythrin (Coulter) and anti-T_8-FITC (Coulter) was performed The cells were adjusted to a final concentration of 2 × 10^6 cells/mL and analyzed by a FACS IV system (Becton Dickinson). OKT4-positive and OKT8-positive cells were sorted under sterile conditions at a rate of ~1,000 cells per second. The sorted cells were used to prepare conditioned media (PHA-T_4CM and PHA-T_8CM) as described for unfractionated cells.

Culture assay for hematopoietic progenitors. Adherent cells were removed from mononuclear bone marrow cells of density <1.077 g/mL in Ficoll to minimize endogenous production of colony-stimulating activities.[10] The nonadherent cells were further depleted of T lymphocytes by immune panning with an anti-E-rosette-receptor antibody.[3]

Hematopoietic colonies were grown as previously described.[4,5] In brief, 1 × 10^5 mononuclear cells/mL in IMDM were immobilized in methylcellulose (0.9%) and cultured with fetal calf serum (FCS, 30%) or human plasma (30%); PHA-LCM or PHA-T_4CM, or PHA-T_8CM or PHA alone, and mercaptoethanol (5 × 10^{-5} mol/L) in humidified atmosphere supplemented with 5% CO_2. After 4 days of incubation, 1 U of erythropoietin (Connaught Step III, Connaught Laboratories, Willowdale, Ontario, Canada) was added to the dishes. The colonies were enumerated with an inverted microscope 12 days after initiation of the cultures.

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RESULTS

Effect of conditioned media on the growth of CFU-GEMM, CFU-C, and BFU-E. Stimulatory activities for multilineage hematopoietic progenitors were prepared either from mononuclear peripheral blood cells or highly purified lymphocytes of helper (T_4) or suppressor/cytotoxic (T_8) phenotype. The sorted cells, prior to PHA stimulation, were reanalyzed by the flow cytometer to demonstrate a purity of >99.5% of T_4 or T_8 cells, respectively. After incubation with PHA, counterstaining of the cultured T_4 (T_8) cells with anti-T_{11}, anti-B_1, anti-MO2, and anti-T_8 (T_4) monoclonal antibodies revealed a positive reaction with >99%, <1%, <1%, and <1% of the cells, respectively.

The conditioned media were tested on nonadherent and T cell-depleted bone marrow cells. Analysis for residual T cells by the peroxidase–antiperoxidase slide technique[11] or the FACS demonstrated <1% of the cells that stained positive for Leu 5, T_{11}, T_4, or T_8 antigen.

The frequencies of CFU-GEMM with increasing concentrations of PHA-LCM, PHA-T_4CM, and PHA-T_8CM are illustrated in Fig 1. The optimal concentrations for all conditioned media were 5% (vol/vol). As shown in Fig 1 and outlined in Table 1, the plating efficiency for CFU-GEMM was significantly higher with the use of PHA-T_4CM (Wilcoxon paired-sample test; $P < .01$) and consistently lower with PHA-T_8CM than with PHA-LCM.

Enhanced growth of committed progenitors, ie, BFU-E and CFU-C, was observed when T_4 or T_8 lymphocyte-conditioned medium was added to the cultures (Table 1). The plating efficiency for BFU-E and CFU-C again was significantly increased with the use of PHA-T_4CM as compared with PHA-LCM (Wilcoxon paired-sample test; $P < .02$ for BFU-E, $P < .02$ for CFU-C). Leukocyte-conditioned medium prepared without the lectin PHA supported BFU-E and CFU-C colony formation; however, CFU-GEMM could not be observed under these culture conditions.

The conditioned media used to study colony formation as outlined in Fig 1 and Table 1 were prepared from 1 × 10^6 cells/mL after stimulation by 1% PHA. The PHA dose–response curves and cell dose–response curves are shown in Fig 2 and Fig 3.

Table 1. CFU-GEMM, CFU-C, and BFU-E Colonies Cultured in the Presence of 30% FCS

Experiment	A	B	C	D	E	F	G	H	I
CFU-GEMM/× 10^5									
—	0	0	0	0	0	0	0	0	0
PHA	0	0	0	0	—	—	—	—	—
LCM	—	—	—	—	0	0	0	0	0
PHA-LCM	6	7	9	9	5	4	5	6	4
PHA-T_4CM	9	11	12	11	9	7	10	10	8
PHA-T_8CM	3	5	3	2	—	—	—	—	—
CFU-C/× 10^5									
—	0	0	—	—	0	0	0	0	0
PHA	4	2	—	—	—	—	—	—	—
LCM	—	—	—	—	80	91	94	123	69
PHA-LCM	101	71	—	—	112	93	80	134	74
PHA-T_4CM	112	88	—	—	137	174	114	139	91
PHA-T_8CM	67	50	—	—	—	—	—	—	—
BFU-E/× 10^5									
—	24	21	—	—	28	18	34	19	27
PHA	25	23	—	—	—	—	—	—	—
LCM	—	—	—	—	49	38	64	59	87
PHA-LCM	58	37	—	—	87	49	79	81	124
PHA-T_4CM	77	53	—	—	106	91	104	112	149
PHA-T_8CM	30	19	—	—	—	—	—	—	—

CFU-GEMM, multilineage colony; PHA, phytohemagglutin; LCM, leukocyte-conditioned medium; CFU-C, granulopoietic colony; BFU-E, erythroid burst, FCS, fetal calf serum.

Conditioned media: 5% vol/vol, PHA 1% vol/vol , dash indicates not done, resp. not evaluated.

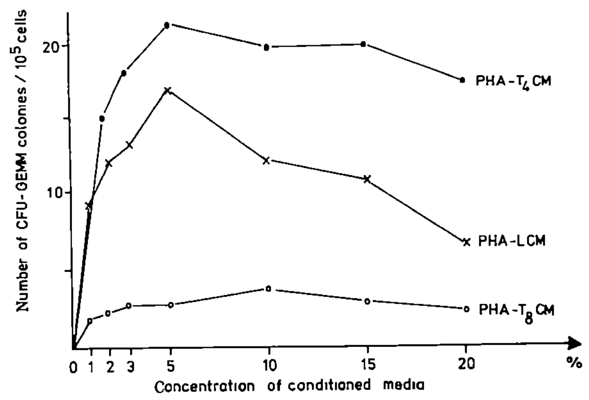

Fig 1. Dose-response curves for phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) (—x—), PHA-T_4CM (—●—) and PHA-T_8CM (—O—) on multilineage hematopoietic colony formation (CFU-GEMM) in the presence of 30% human plasma. Numbers represent the mean of two individual experiments (duplicate cultures).

No colony formation for CFU-GEMM and CFU-C was seen in the absence of conditioned media or when PHA was added to the cultures alone. BFU-E colonies were observed in the presence of exogenously added erythropoietin without conditioned media (Table 1, Figs 2 and 3).

Effect of conditioned media on CFU-M formation. Bone marrow cells obtained from the same donors were plated in plasma from healthy volunteers and from a patient 21 days after bone marrow transplantation. These

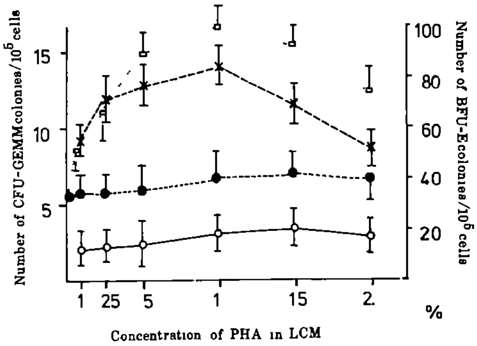

Fig 2. Phytohemagglutinin (PHA) dose–response curves for stimulation of T_4 and T_8 lymphocytes. Bone marrow cells (1 × 10^5/mL) were cultured in the presence of 30% fetal calf serum and 5% of the conditioned media derived from T_4 (T_8) cells (1 × 10^6/mL) and stimulated with 0.1% to 2.0% PHA (three individual experiments). PHA-T_4CM: multilineage hematopoietic colonies (CFU-GEMM) (—x—); erythroid bursts (BFU-E) (· · · · □ · · · ·). PHA-T_8CM: CFU-GEMM (—O—); BFU-E (- - -●- - -).

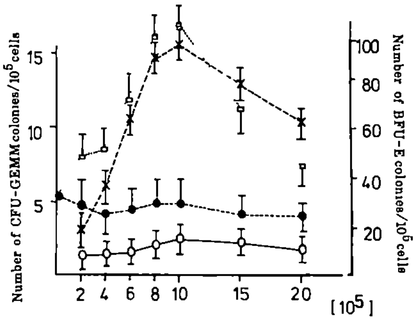

Fig 3. Cell dose-response curves for phytohemagglutinin (PHA) (1%)-stimulated T_4 (T_8) cell-conditioned media. Bone marrow cells (1 × 10^6/mL) were cultured in the presence of 30% fetal calf serum and 5% of the conditioned media, prepared with 2 × 10^5 to 2 × 10^6 T_4 (T_8) cells/mL (three individual experiments). PHA-T_4CM: multilineage colonies (CFU-GEMM) (--x--); erythroid bursts (BFU-E) (····□····). PHA-T_8CM: CFU-GEMM (—○—); BFU-E (---●---).

Table 2. CFU-M and CFU-GEMM Colonies Cultured in the Presence of 30% Plasma From a Posttransplant Patient

Experiment	A	B	C	D	E	F	G
CFU-M/1 × 10^5							
—	8	10	7	10	9	11	8
PHA	9	11	—	—	—	—	—
LCM	—	—	12	10	12	13	14
PHA-LCM	34	37	34	34	27	34	30
PHA-T_4CM	49	54	47	44	38	39	39
PHA-T_8CM	20	21	—	—	—	—	—
CFU-GEMM/1 × 10^5							
—	0	0	0	0	0	0	0
PHA	0	0	—	—	—	—	—
LCM	—	—	0	0	0	0	0
PHA-LCM	11	16	5	6	4	6	4
PHA-T_4CM	19	23	7	9	8	11	6
PHA-T_8CM	3	5	—	—	—	—	—

Conditioned media: 5% vol/vol; PHA 1% vol/vol. Numbers represent the mean of duplicate cultures; (Dash indicates not done) CFU-M, megakaryocytic colonies, other abbreviations as in Table 1.

plasmas were used to replace FCS because plasma provides permissive culture conditions for the growth and differentiation of human megakaryocytic progenitors and of megakaryocytes within mixed multilineage colonies.[6,7]

Dose-response curves for the different conditioned media on CFU-M formation are illustrated in Fig 4. The optimal concentrations for all media were found to be 5% (vol/vol).

The frequency of CFU-M of seven individual experiments is outlined in Table 2. PHA-LCM- and PHA-stimulated media from the T_4 and T_8 subsets supported CFU-M formation. The number of CFU-M colonies was significantly increased with T_4 cell-conditioned medium as compared with PHA-LCM (Wilcoxon paired-sample test; $P < .02$); with PHA-T_8CM, the plating efficiency for CFU-M was much lower.

A small number of CFU-M colonies could be observed when nonadherent and T cell-depleted marrow cells were cultured with posttransplant plasma without the addition of conditioned media (Table 2).

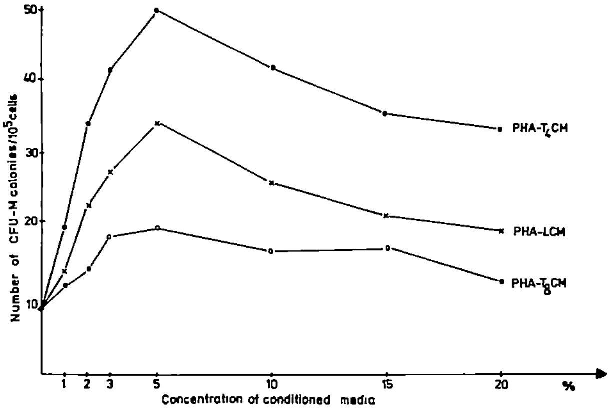

Fig 4. Dose-response curves for phytohemagglutinin leucocyte-conditioned medium (PHA-LCM) (—x—), PHA-T_4CM (—●—) and PHA-T_8CM (—○—) on megakaryocytic colony (CFU-M) formation in the presence of 30% plasma from a posttransplant patient. Numbers represent the mean of two individual experiments (duplicate cultures).

When normal human plasma was used, CFU-M colonies in the absence of conditioned media could be observed only occasionally; in the presence of 5% PHA-LCM, CFU-M colonies ranged between 9 and 22 colonies per 10^5 bone marrow cells ($n = 3$). As in the experiments using posttransplant plasma (Table 2), plating efficiency increased with the use of PHA-T_4CM and was lower in the presence of PHA-T_8CM as compared with PHA-LCM ($n = 3$; data not shown).

The addition of PHA to the cultures did not augment the number of colonies that grew in the absence of conditioned media (Table 3).

Similar results were observed for CFU-GEMM plated with 30% plasma; however, CFU-GEMM colonies were not seen when bone marrow cells were cultured without conditioned media or with PHA alone (Table 2).

DISCUSSION

Cell culture techniques have been established that allow studies of differentiation programs of human CFU-GEMM progenitors. These progenitors can be identified by their ability to form mixed colonies containing basophilic, eosinophilic and neutrophilic granulocytes, erythroblasts, macrophages, megakaryocytes, T cells of different phenotype, and probably B cells.[12-14]

Table 3. Frequency of CFU-M Cultured Without Conditioned Media in the Presence of 30% Plasma From a Posttransplant Patient

CFU-M/1 × 10^5	PHA 0%	PHA 0 05%	PHA 0.5%	PHA 1%	PHA 2%
Exp. 1	8	—	8	9	8
Exp. 2	10	—	12	11	5
Exp. 3	6	5	7	—	—

Plates were incubated with varying concentrations of phytohemagglutinin (experiments represent means of duplicate cultures; dash indicates not done) Abbreviations as in Table 1.

The formation of such multilineage colonies is dependent on the addition of growth-promoting factor(s) provided by a medium conditioned by PHA-stimulated peripheral mononuclear cells (PHA-LCM). Other sources of stimulatory activities for human pluripotent progenitors include human bladder carcinoma cell lines[15,16] and human spleen cells.[17] T lymphocytes are an important source of growth-promoting activities for human committed granulocytic progenitor cells (reviewed in ref. 18), and some T cell lines have recently been shown to release multilineage-CSA.[19,20]

Our study demonstrates that purified T lymphocyte preparations from peripheral blood, defined by anti-T_4 and anti-T_8 monoclonal antibodies, elaborate multiple biological activities (that is, multilineage-CSA, burst-promoting activity, granulocyte-macrophage-CSA, and megakaryocyte-CSA), supporting the growth of noncommitted progenitors (CFU-GEMM) and committed precursors (BFU-E, CFU-C, and CFU-M). Whether the different biological activities provided by the lectin-stimulated T cells are due to a single molecular species such as interleukin-3[2,21,22] or due to multiple colony-stimulating factors is unknown at present.

Our results are in agreement with the published work regarding committed human granulocytic and erythroid progenitors.[23–29] We demonstrated that lymphokines from peripheral T_4 cells consistently increased the plating efficiencies for all progenitors above that seen with supernatants of unseparated mononuclear cells or T_8 cells. This may be related either to an approximate two-fold increase in the number of T_4 positive cells in the preparation for PHA-T_4CM as compared with PHA-LCM or may be explained by the presence of varying levels of inhibitors, such as interferons, in the different conditioned media.

The elaboration of growth-promoting activities for CFU-GEMM is critically dependent on the activation of T cell subsets by PHA. Conditioned media prepared without mitogen supported BFU-E, CFU-C, and CFU-M colony formation; however, the number of colonies were significantly lower than with supernatants derived from PHA-activated cells.

The sorting of T lymphocyte subsets by FACS and subsequent incubation of these cells with PHA revealed cells with a purity of >99%. Several studies suggest that monocytes and T cells can collaborate to stimulate the growth of CFU-C and BFU-E.[30–33] Our studies cannot rule out this possibility, but with a number of nonlymphocytic cells of <1% in the final cell suspension used to prepare the conditioned media, the presence of autologous monocytes does not seem to be required for the in vitro production of lymphokines

Our data show that nonadherent and T cell-depleted bone marrow cells are unable to support colony growth alone, since CFU-GEMM and CFU-C were not observed when the target cells were cultured without conditioned media or with PHA alone. BFU-E and CFU-M colony formation in the absence of (PHA-)LCM can be attributed to burst-promoting activity present in serum[34] or in the unpurified erythropoietin preparation used[5] and to megakaryocyte-CSA in human plasma prepared from posttransplant patients.[35] Further evidence that PHA-LCM contains factor(s) that directly stimulate progenitor cell growth and differentiation has been provided by a single-cell culture system that excluded cell–cell interactions and eliminated the endogenous production of factors by contaminating macrophages and lymphocytes.[36] It cannot be excluded, however, that the conditioned media may stimulate accessory cells other than adherent cells or T lymphocytes to induce secondary factor(s) important in stimulating colony formation.

In summary, our data provide evidence that human T lymphocyte subpopulations elaborate lymphokine(s) for the growth and differentiation of noncommitted and committed hematopoietic progenitors. The availability of conditioned media from highly purified cell populations may facilitate studies of events in early hematopoiesis, and these cells may be useful in the molecular cloning of human multi-CSF.

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REFERENCES

1. Nabel GJ, Greenberger S, Sakakeeny MA, Cantor H: Multiple biologic activities of a cloned inducer T-cell population Proc Natl Acad Sci USA 78:1157, 1981

2. Rennick DM, Lee FD, Yokota T, Arai KI, Cantor H, Nabel GJ: A cloned MCGFcDNA encodes a multilineage hematopoietic growth factor· multiple activities of Interleukin 3. J Immunol 134:910, 1985

3. Fauser AA, Kanz L, Bross KJ, Löhr GW: T-cells and probably B-cells arise from the malignant clone in chronic myelogenous leukemia. J Clin Invest 75:1080, 1985

4. Fauser AA, Messner HA: Granulo-erythropoietic colonies in human bone marrow, peripheral blood, and cord blood. Blood 52:1243, 1978

5. Fauser AA, Messner HA: Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts Blood 53:1023, 1979

6. Kanz L, Straub G, Bross KG, Fauser AA: Identification of human megakaryocytes derived from pure megakaryocytic colonies (CFU-M), megakaryocytic-erythroid colonies (CFU-M/E), and mixed hematopoietic colonies (CFU-GEMM) by antibodies against platelet associated antigens Blut 45:267, 1982

7. Messner HA, Jamal N, Izaguirre C: The growth of large megakaryocyte colonies from human bone marrow. J Cell Physiol 1·45, 1982 (suppl)

8. Kanz L, Löhr GW, Fauser AA: Interference of a human leukocyte interferon preparation with stimulatory activities in leukocyte-conditioned medium for human hematopoietic stem cells. Int J Cell Cloning 2:286, 1984.

9 Fauser AA, Cooper BA, Kanz L, Löhr GW. Interaction of 3-deaza-adenosin, a phospholipid methyltransferase inhibitor, on the production of pluripoietins from human peripheral T-cells. Exp Hematol 13.299, 1985

10 Messner HA, Till JE, McCulloch EA. Interacting cell populations affecting granulopoietic colony formation by normal and leukemic human marrow cells. Blood 42:701, 1973

11. Bross KJ, Pangalis GA, Staatz CG, Blume KA: Demonstration of cell surface antigens and their antibodies by peroxidase–anti-peroxidase method. Transplantation 25:221, 1978

12. Fauser AA, Löhr GW: Recloned colonies positive for T-

cell-associated antigens derived from mixed hemopoietic colonies (CFU-GEMM). Proc Soc Exp Biol Med 170 220, 1982

13. Messner HA, Izaguirre CA, Jamal N· Identification of T-lymphocytes in human mixed hemopoietic colonies. Blood 58.402, 1981

14. Fauser AA, Kanz L, Löhr GW: Identification of B-cells in multilineage hematopoietic colonies derived from cells of patients with lymphocytic lymphoma Proc Natl Acad Sci USA 82:883, 1985

15. Platzer E, Welte K, Lu L, Gabrilove J, Mertelsmann R, Moore MAS: Purification and biological characterization of human pluripotent colony-stimulating factor. Exp Hematol 12:415, 1984

16. Myers CD, Katz FE, Joshi A, Millar JL. A cell line secreting stimulating factors for CFU-GEMM culture. Blood 64:152, 1984

17. Fabian I, Douer D, Levitt L, Kletter Y, Greenberg PL: Human spleen cell generation of factors stimulating human pluripotent stem cell, erythroid, and myeloid progenitor cell growth. Blood 65:990, 1985

18. Burgess AW, Metcalf D: The nature and action of granulocyte-macrophage colony stimulating factors. Blood 56.947, 1980

19. Fauser AA, Messner HA, Lusis AY, Golde DW: Stimulatory activity for human pluripotent hemopoietic progenitors produced by a human T-lymphocyte cell line. Stem Cells 1:73, 1981

20. Greenberger JS, Krensky AM, Messner H, Burakoff SJ, Wandl U, Sakakeeny MA· Production of colony-stimulating factor(s) for granulocyte-macrophage and multipotential (granulocyte/erythroid/megakaryocyte/macrophage) by clonal lines of human IL-2-dependent T-lymphocytes. Exp Hematol 12.720, 1984

21. Hapel AJ, Fung MC, Johnson RM, Young IG, Johnson G, Metcalf D. Biologic properties of molecularly cloned and expressed murine Interleukin-3. J Immunol 134:910:1985

22. Welte K, Platzer E, Lu L, Levi E, Mertelsmann R, Moore MAS. Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. Proc Natl Acad Sci USA 82·1526, 1985

23 Torok-Storb B, Martin PJ, Hansen JA: Regulation of in vitro erythropoiesis by normal T-cells: Evidence for two T-cell subsets with opposing function. Blood 58·171, 1981

24. Mangan KF, Chikkappa G, Bider LZ, Scharfman WB, Parkinson DR: Regulation of human blood erythroid burst forming unit (BFU-E) proliferation by T-lymphocyte subpopulations defined by Fc receptors and monoclonal antibodies. Blood 59:990, 1982

25. Platzer E, Welte K, Broxmeyer H, Mertelsmann R: OKT3 monoclonal antibody induced production of interleukin 2 and colony-stimulating factor. Exp Hematol 11:141, 1983 (suppl 14)

26. Koizumi S, Yamagami M, Horita S, Miura M, Sano M, Hentor N, Tarigudri N· Effects of OKT3$^+$, OKT4$^+$ and OKT8$^+$ T-cell subsets on steady-state granulopoiesis in vitro. Int J Cell Cloning 1.130, 1983

27. Hesketh PJ, Sullivan R, Valeri CR, McCaroll LA: The production of granulocyte-monocyte colony-stimulating activity by isolated human T-lymphocyte subpopulations. Blood 63·1141, 1984

28. Linch DC, Nathan DG: T-cell and monocyte derived burst promoting activity directly act on erythroid cells. Nature 312:775, 1984

29. Wisniewski D, Strife A, Wachter M, Clarkson B: Regulation of human peripheral blood erythroid burst forming unit. Growth by T-lymphocytes and T-lymphocyte subpopulations defined by OKT4 and OKT8 monoclonal antibodies Blood 65:456, 1985

30. Zuckerman KS. Erythroid burst forming units: Growth in vitro is dependent on monocytes, but not T-lymphocytes. J Clin Invest 67:702, 1981

31. Reid CDL, Baptista LC, Chanarin I. Erythroid colony growth in vitro from human peripheral blood null cells: Evidence for regulation by T-lymphocytes and monocytes. Br J Haematol 48:155, 1981

32. Verma DS, Spitzer G, Beran M: DR antigen positive monocyte-macrophages control granulocyte-macrophage colony-stimulating activity and burst promoting activity elaboration in man Am J Hematol 12:403, 1982

33. Bagby GC, Rigas UD, Bennett RM, Vandenbark AA, Garewall HS: Interaction of lactoferrin, monocytes, and T-lymphocyte subsets in the regulation of steady-state granulopoiesis. J Clin Invest 68:56, 1981

34. Iscove NN: Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin-stimulated mouse spleen cells, in Golde DW, Cline MJ, Metcalf D, Fox CF (eds)· Hematopoietic Cell Differentiation (ICN-UCLA Symposia on Molecular and Cellular Biology, Vol 10). Academic Press, Orlando, Fla 1978, p 37

35. Solberg LA, Messner HA· Megakaryocyte colony stimulating activity (MEG-CSA) in the plasma of bone marrow transplant recipients. Blood 62:147a, 1983 (suppl 1)

36. Nakahata T, Tsuji K, Ishiguro A, Ando O, Norose N, Koike K, Akabone T: Single-cell origin of human mixed hemopoietic colonies expressing various combinations of cell lineages. Blood 65:1010, 1985

A Prospective Study of The Value of Bone Marrow Erythroid Progenitor Cultures in Polycythemia

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The diagnostic value in polycythemia of the presence of endogenous erythroid colonies derived from bone marrow cells (EECs) was assessed in a prospective study on 108 patients referred for polycythemia (Hb > 18 g/dL in men, > 16 g/dL in women) with normal plasma volume by comparison with the standard criteria, the bone marrow grade, and the serum erythropoietin (Epo) level. Total red cell volume (TRCV) was high (>36 mL/kg in men, 32 mL/kg in women) in 87 cases (group A) and slightly increased in 21 cases (group B). Standard criteria were applicable in 63 of 108 cases (57%); 46 were PV and 17 were secondary polycythemia (SP). Standard criteria were nonapplicable in 45 cases. EECs were present in 65 cases (60%) with a ratio of EEC/Epo-stimulated colonies of 39.5% ± 18% (extremes 10% to 80%). EECs were noted in 43 of 46 polycythemia vera (PV) and 0 of 17 SP. Among the 45 unclassified cases, EECs were noted in 22: 18 of 29 cases from group A (10 with 2 major and 1 minor criteria; 8 with 2 major criteria) and 4 of 16 cases from group B (with variable standard criteria, 2 belonging to a PV family). In group A, there was a positive significant correlation between EECs and the presence of two major and 1 minor criteria ($P < .0001$). In group B, there was a positive significant correlation between EECs and the presence of at least 1 major criterion and 2 minor criteria or a family background ($P < .0001$). The unclassified polycythemias with EECs in the bone marrow are characterized by a bone marrow grade and a mean serum Epo level not different from that of patients with PV and an active course of the disease. The unclassified polycythemias without EECs in the bone marrow are a heterogenous group corresponding in some cases to SPs of unknown origin (slightly increased bone marrow grade and/or high serum Epo level), and in others cases to spurious polycythemias (normal bone marrow grade and/or normal Epo level). In conclusion, EECs were of great value in differentiating PV from SP ($P < .001$), and in allowing the diagnosis of PV in the absence of all the standard criteria even when TRCV was slightly increased. In our study, EEC improved the classification of polycythemia by 22%. The recommended diagnostic steps for the evaluation of polycythemia must be reconsidered.

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POLYCYTHEMIA is a descriptive term that simply denotes an elevated hemoglobin and hematocrit. As previously described,[1] polycythemia could be classified into three groups: polycythemia vera (PV) and secondary polycythemia (SP) from increased production of erythropoietin (Epo); if the underlying cause of this abnormal production is eliminated, the excess RBCs disappear. These first two groups represent an absolute polycythemia, ie, an abnormal increase in the total volume of circulating RBCs (TRCV). The third group, relative or spurious polycythemia, is characterized by an elevated hematocrit but has only a normal or slightly increased TRCV.

PV is a chronic myeloproliferative disorder characterized by an elevated TRCV and usually leucocytosis, thrombocytosis, and splenomegaly. The Polycythemia Vera Study Group (PVSG) has developed a useful set of clinical criteria[2] for the diagnosis and the treatment of PV. The classification of polycythemia and the set of criteria of PVSG have two disadvantages, however. First, the classification into three groups excludes many who have only a true increase of TRCV and absence of an underlying cause, pure erythrocytosis (PE),[3] or so-called idiopathic erythrocytosis.[4] Second, some individuals who do have PV will be excluded by these criteria, mainly at an early stage.

The demonstration in 1974 by Prchal and Axelrad[5] that erythroid colonies derived from bone marrow cells of PV can develop in a semisolid culture medium without the addition of Epo (EECs) marked an important turning point. Although EECs have been observed in other diseases as well, including erythroleukemias,[6,7] some cases of sickle cell anemia,[8] and myeloproliferative diseases other than PV,[5,9-11] their presence in polycythemia could, for the first time, constitute a characteristic sign for the diagnosis of PV. EECs were always reported in PV and never in SP.[5,12-14]

To determine the diagnostic value of the presence or absence of EECs in polycythemias, we made a prospective study on 108 patients with suspected polycythemia, comparing the standard criteria with the results of bone marrow cultures, with or without Epo. Our aim was to distinguish PV from SP and to recognize PV in the possible absence of all the standard criteria, hoping to simplify and improve the diagnosis of PV.

MATERIALS AND METHODS

Patients. This study was made of 108 patients (69 men, 39 women) with a mean age of 59.4 ± 17 years (19 to 91 years) referred for suspected polycythemia to the department of hematology of the Saint Antoine Hospital between 1979 and 1985. The question of polycythemia was raised when the hemoglobin exceeded 18 g/dL in men and 16 g/dL in women All patients with relative polycythemia from reduced plasma volume were excluded. None had received treatment before the study. Although familial polycythemia vera is rare,[15,16] 5 patients belonged to 3 families in which 1 parent and 1 child have this disease.

Criteria. The criteria established by the PVSG were used in all the cases except for LAP scoring and serum B_{12} estimations, which were made only when the other criteria were absent; bone marrow

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cultures were made prior to treatment. Bone marrow biopsy was performed in 56 patients. Epo level in serum was measured in 54 cases. When necessary, special studies were done to find the cause of polycythemia: blood oxygen affinity; hemoglobin electrophoresis; carboxy-hemoglobin evaluation; kidney echographic examination; and cerebral, abdominal, and thoracic computerized tomographic scans. All patients were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

The TRCV was measured by the ^{51}CR-labeled RBC dilution method.[17] TRCV was considered abnormally elevated when it was >32 mL/kg in women (normal 25 mL/kg) and 36 mL/kg in men (normal 30 mL/kg).

WBC counts, platelet counts, and arterial oxygen saturation were determined by standard techniques. LAP scoring was based on the Rutenberg method.[18] Serum B12 estimations were performed by radioisotope dilution.[19] Spleen volume was determined by physical and echographic examination. Percutaneous trephine biopsies were taken from the anterior iliac crest with Tanzer or Jamshidi needles. Sections of marrow were prepared and stained by routine techniques described elsewhere.[20] Each histological section was examined independently by two histopathologists Examination was done under a total magnification of 400x using a Zeiss microscope. An area of 16 to 90 cross-sections was measured (mean 31). The cellularity was subjectively evaluated in the same microscopic fields as those in which the number of megakaryocytes was counted Cellularity was graded as described elsewhere[21]: normal, 40% to 49%; slight increase, 50% to 59%; moderate increase, 60% to 89%; marked increase, 90% to 100%. Megakaryocyte concentration was evaluated by the mean of the number of megakaryocytes counted in all the cross-sections for each patient. A normal concentration generally ranged from one to two megakaryocytes per field at that magnification (400x). The grade of megakaryocytes was: normal, 1 to 2; slight increase, 2 to 3; moderate increase, 3 to 6; marked increase, 6 to 12. The final grading was established by comparing the results of cellularity grade and megakaryocyte concentration

Epo assay Epo activity was determined in vitro by incorporation of radioactive iron into cultured fetal mouse liver cells[22] Embryos were harvested from 13-day-pregnant Balb/c mice (Charles River, France). The mechanically dissociated liver cells were suspended in RPMI 1640 medium at 1.6 × 10^6 cells/mL containing 7% fetal calf serum (FCS), 85 μmol/L of bovine serum albumin, and 0 4 μmol/L of human transferrin. Cell suspension was distributed in a small volume (200 μL)[23] into the wells of Nunclan culture plates. After incubation for 21 hours, human ^{59}Fe-transferrin was added, followed by 5 hours of additional incubation. Epo fraction prepared from whole patient serum[24] was used in the assay and calibrated against a standard of pig serum Epo (Centre National de Transfusion Sanguine, Paris) referred to as the second international reference preparation (courtesy of the WHO laboratory for biological standards, Mill Hill, London) and NIH standard (specific activity: 1,546 UI/A 280) generously donated by E. Goldwasser. Each sample was cultured at three or four protein concentrations and in four replicate assays, and results were expressed in milliunits per milliliter: mean ± SEM· 14 ± 4 for normal human serum.

Bone marrow culture. Bone marrow aspirate (1.2 mL) was collected in 200 U of sterile preservative-free heparin. Mononuclear cells, isolated by using Hypaque-Ficoll (density 1.077) were washed three times with Iscove-modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY).[25] The nucleated cells collected were plated at a final concentration of 2.5 x 10^5/mL by the plasma clot method described by MacLeod[26] in 30 × 10-mm Petri dishes containing 20% heat-inactivated FCS, 1% deionized bovine serum albumin (BSA, Sigma, St Louis) prepared according to the method of Worton and colleagues,[27] CaCl 34 mL, thrombin 1 U/mL, citrated beef plasma (GIBCO) 10%, with or without 1 U/mL Epo (step III, Connaught, Toronto). All tests were done at least twice. Clots were incubated at 37 °C in 5% CO_2. After 7 days of incubation, the clots were stained with benzidine and hematoxylin to determine the number of colonies with hemoglobin that had formed. Each clot was examined under 100x magnification, and only colonies consisting of eight or more benzidine-positive cells were counted. These colonies were derived from CFU-E that normally proliferate and differentiate into proerythroblasts in response to Epo. The presence of erythroid colonies that grew without the addition of exogenous Epo was defined as a positive culture; the occurrence of erythroid colonies only in the presence of exogenous Epo was defined as a negative culture.

Statistical methods. All results are given as means ± SE. The Wilcoxon Mann-Whitney rank-sum test was used to compare erythropoietin levels from patients with or without endogenous erythroid colonies. The analysis of variance (ANOVA) test was used to compare the means of the level of erythropoietin among patients with PV, with SP, with UP and EECs, and with UP without EECs The Chi-square test was used to analyze contingency tables of patients with and without EEC obtained by several cut-offs: presence or not of standard criteria, and grade of the bone marrow.

RESULTS

Standard criteria. The main clinical and laboratory data from the 108 patients are summarized in Table 1. The TRCV delineated two groups: group A, consisting of 87 patients (50 men, 37 women) in which it was >36 mL/kg (49 ± 12.5 mL/kg) in men and >32 mL/kg (42 ± 9 5 mL/kg) in women; and group B, consisting of 21 patients (19 men, 2 women) with a TRCV between 30 and 36 mL/kg (32.8 ± 2.1 mL/kg) in men, and 27.8 and 28.6 mL/kg for the two women, respectively. None of these 21 group B patients had a reduction of plasma volume; therefore, there was no hemoconcentration. None was obese or had phlebotomies prior to diagnosis. The mean corpuscular volume of the RBCs was normal in each case of this group.

According to the criteria of the PVSG, 46 of the 87 patients of group A had PV (mean TRCV: 49 ± 11.5 mL/kg). Twelve patients from group A and 5 of group B had SP:

Table 1. Clinical and Biological Data of Patients With Suspected Polycythemia

Data	Group A	Group B
No. of patients	87	21
Total (%)	100	100
Sex		
M	58	90
F	42	10
Sex ratio (M *v* F)	1 41/1	NS
Arterial oxygen saturation 92%	93	100
Splenomegaly	33	15
Leucocytosis ≥12 × 10^9/L	39	20
Thrombocytosis ≥400 × 10^9/L	44	15

Group A is defined by a total volume of circulating RBCs (TRCV) ≥ 36 mL/kg in males and TRCV ≥ 32 mL/kg in females Group B is defined by a TRCV between 30 and 36 mL/kg in males and between 25 and 32 mL/kg in females.

7 from chronic respiratory insufficiency, 3 from smoking excess, 4 from hepatomas, 1 from adrenal cancer, 1 from renal cancer, and 1 from essential thrombocythemia. Forty-five patients (29 of group A and 16 of group B) lacked the criteria for PV or SP and were considered to have unclassified polycythemia (UP).

Culture of bone marrow erythroid progenitors. In 65 of 108 cases (60%), colonies derived from CFU-E were observed without addition of Epo (EECs): 61 of 87 cases (70%) of group A and 4 of 21 cases (20%) of group B. In the remaining 43 cases, no growth occurred in the absence of Epo. The mean of EECs was 213 ± 334 colonies for 2.5×10^5 cells plated with a range from 12 to 1,455 colonies. In 34 cases, the number of colonies was also counted at the optimal concentration of 1 U/mL Epo: the mean was 439 ± 621 colonies for 2.5×10^5 cells plated with a range from 69 to 2,255 colonies. To appreciate the importance of the abnormal clonal population in relation to total erythroid progenitors, in these 34 cases we counted and compared the number of EECs with the number of colonies stimulated by Epo in the concentration of 1 U/mL. The ratio of EECs to Epo-stimulated colonies was 39.5% ± 18% (range 10% to 80%).

Comparative study of standard criteria and presence or absence of EECs. In group A (Table 2), 43 of 46 patients (93%) with PV had positive cultures. Three, in spite of fitting the standard criteria, had negative cultures. The first two were technical failures. In all three cases, the cultures could not be repeated. Two of these patients have died, one from blastic transformation of PV, the second from an astrocytoma. None of the 12 patients with SP had a positive culture. For diagnosis of PV, there was an excellent statistical correlation ($P < .0001$) in this group of 46 PV and 12 SPs between the standard criteria and positive cultures. The study of the 29 patients with unclassified polycythemia showed positive cultures in 18 cases (62%): 11 of 12 patients (6 men, 5 women) with 2 major and 1 minor criterion, 7 of 17 patients (4 men, 3 women) with 2 major and no minor criteria, and one of whom belonged to a PV family. In all group A, the presence of at least of 2 major criteria and 1 minor criterion makes the probability of the culture being positive statistically significant ($P < .0001$).

In group B, 4 patients (3 men, 1 woman) (20%) had positive cultures: one with 2 major and 2 minor criteria who belonged to a PV family, one with 2 major criteria, one with 1 major criterion and 2 minor criteria, and the last one with only 1 major criterion but with a positive family history. The 5 patients with a disposition toward polycythemia had negative cultures. The remaining 12 had negative cultures.

In group B, the presence of at least 1 major criterion and 2 minor criteria or a family background makes the probability of the culture being positive statistically significant ($P < 0.0001$), in spite of the small size of the series ($n = 21$).

Thus, the culture of bone marrow erythroid progenitors has allowed differentiation among the 45 unclassified polycythemias (UP), 22 cases with EECs (18 cases from group A and 4 from group B), and 23 cases without EECs (11 from group A and 12 from group B).

To evaluate the significance of the presence of EECs in the absence of criteria for PV, we studied other laboratory tests that can help in the diagnosis of PV and the prognosis of the disease of these patients.

Unclassified polycythemias with EECs in the bone marrow. The 22 patients were 13 men and 9 women with a mean age of 56.3 ± 15.6 years (range 19 to 81 years); 18 of 22 have had in common a high TRCV (mean 48 ± 13 mL/kg), a normal arterial oxygen saturation, and the absence of splenomegaly. The WBCs count was $>12 \times 10^9$/L in three cases, and the platelet count was $>400 \times 10^9$/L in eight cases. The LAP score was elevated in one case, but became high in two other cases during progression of the disease. Four of 22 have had a slightly increased TRCV (mean 32.6 ± 2.6 mL/kg) with a normal arterial oxygen saturation. Splenomegaly was present in two cases. WBCs and platelets were elevated in two cases.

Bone marrow biopsies were done in 15 of 22 patients. The results are shown in Table 3. The grade of the bone marrow was normal in 3 cases, slightly increased in 1 case, and

Table 2. Comparative Study of Standard Criteria of Polycythemia and Results of Bone Marrow Erythroid Progenitor Cultures

Group	Standard Criteria	EECs+
A		
Polycythemia vera	46	43 (93%)
Secondary polycythemia	12	0
Unclassified polycythemia	29	18 (62%)
Total	87	61 (70%)
B		
Secondary polycythemia	5	0
Unclassified polycythemia	16	4 (25%)
Total	21	4 (20%)

EECs, endogenous erythroid colonies derived from bone marrow cells developing without exogenous erythropoietin.

Standard criteria, criteria proposed by the Polycythemia Vera Study Group and criteria of secondary polycythemia Group A is defined by a TRCV of ≥ 36 mL/kg in males and TRCV ≥ 32 mL/kg in females; group B is defined by a TRCV between 30 and 36 mL/kg in males and between 25 and 32 mL/kg in females.

Table 3. Grade of Bone Marrow in Patients With Polycythemia Vera and With Unclassified Polycythemias With or Without Endogenous Erythroid Colonies

Bone Marrow (Grade)	EECs+		EECs−	
	PV	UP (A + B)	UP (A)	UP (B)
N	0	3	3	5
+	1	1*	3†	1
++	7	6	1	—
+++	14	5	—	—
Total	22	15	7	6

UP, unclassified polycythemias.

Grade of bone marrow cellularity N, normal, +, slight increase; ++, moderate increase; +++, marked increase. A, patients with a total volume of circulating RBCs (TRCV) >36 mL/kg in males and >32 mL/kg in females; B, patients with a TRCV between 30 and 36 mL/kg in males and between 25 and 32 mL/kg in females. All numbers indicate number of cases.

*Patient had a normal number of megakaryocytes.

†One-third had a normal number of megakaryocytes.

increased in 11 cases. There was no difference between the patients who lacked one or more standard criteria. In the meantime, 22 bone marrow biopsies were done among the 46 patients with PV: the grade of the bone marrow was normal in one case and increased in 21 cases. When a cut-off point between normal and slightly increased cellularity on one hand and moderate or marked cellularity on the other hand was chosen, there was no difference between the two groups (NS).

Epo level in the serum was measured in 12 of 22 patients: the results are presented in Fig 1. The Epo level was low in 5 cases, normal in 6 cases, and high in only 1 case. The mean level was 11.75 ± 6.78 mU/mL of serum, ranging from 4 to 30 mU/mL. There was no difference between the patients who lacked one or more standard criteria. Meanwhile, Epo levels were measured also in 16 patients with PV: they were low in 5 cases, normal in 9 cases, and high in 2 cases. The mean level in this group was 13.5 ± 10.5 mU/mL of serum, ranging from 4 to 42 mU/mL. The comparison between the two groups has not shown any statistically significant difference (NS).

The course of the disease could be followed in 21 of 22 cases with a mean follow-up of 4.25 ± 2 years (extremes: 1.5 to 7 years).

Sixteen of 18 patients of group A were treated. In 15 cases, the disease was continuously active with relapses under treatment. In three of these cases, PV became evident on the standard criteria during follow-up. Thrombophlebitis occurred in five cases. Five patients died: one from agnogenic metaplasia after a course of 4 years, and four from vascular thrombosis. One patient had a stable disease under treatment for 4 years, and the patient who was not treated but followed had a permanent and nonevolving polycythemia.

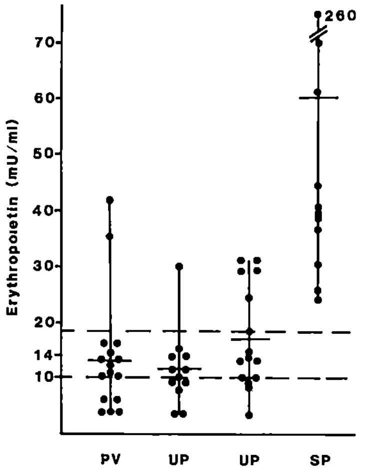

Fig 1. Erythropoietin (Epo) levels in the serum of patients with polycythemia. PV, polycythemia vera (mean = 13.5 ± 10.5 mU/mL); n = 16. UP (EECs +), unclassified polycythemia with EECs, endogenous erythroid colonies derived from bone marrow cells developing without exogenous erythropoietin, in the bone marrow (mean = 11.75 ± 6.78 mU/mL); n = 12. UP (EECs −), unclassified polycythemias without EECs in the bone marrow (mean = 17.16 ± 9.25 mU/mL); r = 15 and SP, secondary polycythemias (mean = 61 ± 6.8 mU/mL), n = 11. The level of Epo in the serum of normal controls was 14 ± 4 mU/mL (between dotted lines). The analysis of variance between the four groups of patients showed a statistically significant difference ($P < .05$). Solid bars represent the mean of Epo concentrations in each group.

The four patients with a slightly increased TRCV received treatment and achieved complete remission. Two patients have had an active disease with an increasing TRCV, and one died from agnogenic metaplasia after a course of 6 years. The two other patients are always in complete remission, under treatment.

Unclassified polycythemias without EECs in the bone marrow. All but one of the 23 patients were men, with a mean age of 49 ± 15.5 years (range 22 to 76 years). They have had in common an absence of splenomegaly, normal arterial oxygen saturation, normal WBC and platelet counts; the LAP scores and the B-12 serum levels were also normal. Eleven patients have had a clearly increased TRCV (mean 40 ± 3.4 mL/kg) whereas the 12 others have had a slightly increased TRCV (mean 32.8 ± 2.1 mL/kg).

Bone marrow biopsy was done in 13 of 23 cases. The results are shown in Table 3. The grade of the bone marrow was normal in 8 cases, slightly increased in 4 cases, and increased in 1 case. There was a striking difference between the bone marrow in these patients and the patients with PV ($P < .01$) as well as the patients with unclassified polycythemias with EECs ($P < .01$).

Epo level was measured in 15 of 23 cases (Fig 1). It was low in two cases, normal in eight cases, and high in five cases, with a mean of 17.16 ± 9.25 mU/mL and a range of values from 4 to 31 mU/mL. Among the patients with high levels, three had increased TRCV (group A) and two had a slightly increased TRCV (group B). None of these patients had tumors; blood oxygen affinity was studied in three cases and was normal. In two cases, excessive smoking was suspected but the level of carboxyhemoglobin was not evaluated. In all five patients, the significance of the high level of erythropoietin remains to be found. There is no significant difference between this group and patients with PV or with UP and EEC (NS). There is a statistically significant difference, however, between this group and a group of 11 SPs studied in the meantime ($P < .001$).

All these patients but one were followed in absence of treatment with a mean follow-up of 3.25 ± 1.5 years (range 1.5 to 7 years). There were no significant changes in the blood counts, and no complications occurred.

DISCUSSION

Two observations stand out in this study of an unselected series of patients with different forms of polycythemia. First, the standard criteria for the etiologic diagnosis were applicable in only 63 of 108 (57%) of the cases: 46 PV and 17 secondary type. If one considers the group with definitely abnormal TRCV, the etiologic diagnosis was possible in 57 of 87 (66%) of the cases.

Second, the presence of an abnormal population of erythroid progenitors was observed not only when all the standard criteria of PV were present, but also (in many cases) when they were lacking. In group A, the culture was positive in 93% of patients having all the criteria of PV and in 63% in whom some of these criteria were missing. In group B, the culture was also positive in 20%.

The criteria proposed by the PVSG in 1965 have had the merit of distinguishing a homogenous group of patients with PV but lead to the exclusion of atypical and early forms.[28] Therefore, an important number of polycythemias could not be classified as PV even though the TRCVs were very elevated: 29 of 87 cases in our series. Moreover, the definition of PV as a value of TRCV >2 SD (+2 SD) of the average value likewise contributed to the exclusion of definite cases with abnormally high blood counts, a moderate elevation of TRCV, and no reduction of total plasma volume. These cases, classified by some as spurious polycythemias,[1] may equally well be forms of PV at a stage just starting or smoldering cases of PV. The long absence of a specific measurable criterion for a primary disorder involving the production of the erythroblastic line explains these difficulties.

Although the initial cause of PV remains unknown, abnormality of the erythroblastic line is now well established through recent findings. PV can be considered the result of an abnormality of a clone from myeloid stem cells[29] from which a population of erythroid progenitors recognized by its behavior in in vitro semisolid medium derives.[5]

The diagnostic value of the presence of this population during the course of polycythemia has not been the object of important prospective studies, as far as we know. The absence of spontaneous erythroid colonies in SP has been reported by many authors. Clement and colleagues[30] reported finding spontaneous colonies in 8 of 18 patients with pure erythrocytosis, a debatable term used for cases characterized by an important and isolated increase in TRCV. Analysis of our results indicates that the finding of spontaneous colonies is of great value in differentiating PV from secondary forms ($P < .001$); in PV, the cultures were positive in all but three cases, whereas they were negative in all the secondary types. In the three negative cultures seen in our series of PV, two were due to technical failure. The third case, that of a 17-year-old male who died after a 2-year course of a cerebral astrocytoma, without a repeat bone marrow culture, was more problematic. The presence of EECs in patients with polycythemia in the absence of all the standard criteria for PV raises the question of their use as a diagnostic tool for the classification of polycythemias. One may assume that finding an abnormal population of EECs in the course of a polycythemia justifies the diagnosis of PV.

We believe that data in our study may ascertain this hypothesis. First, there is a strong positive correlation between the presence of EECs and the grade of the bone marrow. Bone marrow biopsy is an important diagnostic test (31–33) to recognize PV and distinguish it from SP or spurious polycythemias.

Hypercellularity and an increase in the number of megakaryocytes are the two main features in PV.[21,33] In our study, a striking difference is noted between unclassified polycythemias with or without EECs. When EECs are present, the bone marrow has the same appearance as in PV in 11 of 15 patients. Because normal or slightly increased bone marrow cellularity was also described in PV,[21] one cannot exlude the four other patients. One of them has had a very active disease. Two were treated and until now have had a quiet course. Only one was not treated and has had a stable polycythemia. Conversely, when EECs were not present, 12 of 13 cases had a normal or slight hypercellular bone marrow, as in SP or spurious polycythemias.[32]

Second, there is no difference between the level of erythropoietin in the serum of patients with PV and unclassified polycythemias with EECs. The means of the two groups are in the normal range. In PV, Epo level in the serum is generally low[34] or in the normal range,[35] whereas it is high in SPs.[34–36] Discrepancies can be noted, however, despite the method of dosage used, which reduces the diagnostic importance of this test for individuals. In one patient with UP and EECs, the level was as high as it was in two patients with PV. High levels of Epo were also observed in PV by Koeffler and Goldwasser[35] with a radioimmunological method. Conversely, there was a greater proportion of patients (5 of 15) with high Epo levels in the group with unclassified polycythemias without EECs.

The cause of the increase that produced a true polycythemia in three patients and a slight increase of TRCV in the two others was not found until now. The higher mean of Epo in this group, although not significantly different from the mean of Epo in PV or in patients with EECs, leads to the consideration of these patients as a heterogenous population. Some of them may have SPs, some real unclassified polycythemias, and some may have spurious polycythemias.

Finally, the course of the disease in the patients with unclassified polycythemias with EECs was very similar to that of the patients with PV. Fifteen of 21 patients who could be followed have had an active disease which has required treatment. Several cases of thrombophlebitis were noted. Six patients died from vascular complications and from myelofibrosis, as happens in PV. Conversely, the patients with unclassified polycythemias without EEC have had a quiet course, without treatment.

We believe that the varying combinations of the hematological data in the patients with polycythemia and EECs are related to the diversity of lineage involvement in the chronic myeloproliferative syndromes, the reasons for which have been unclear.[37–39] These varied manifestations may be dependent on the predominant impaired stem cell and its proliferative strength. Thus, the 18 of 29 cases of unclassified polycythemias of group A were PV with variable hematologic findings. Of the four patients of group B with positive cultures, three had all the standard criteria of PV except for a sufficiently high TRCV. The absence of this first criterion is easily explained if it concerns a beginning case or one that has hardly evolved. The fourth patient had only one major criterion but had a parent with PV. The rarity of familial forms of PV, which represent only 0.8% of all PV cases, is an important argument in linking this case to a beginning stage of PV, all the more so because the hemoglobin and hematocrit were elevated and the TRCV slightly increased without any decrease in plasma volume.

The unclassified polycythemias without EECs were a heterogenous group. The cause of the polycythemias in the patients with a high TRCV (group A) remains to be found, especially for the patients with a normal Epo level.

The 12 cases of group B cannot be better defined for the moment. The patients with normal bone marrow biopsies and normal Epo levels were perhaps normal subjects corresponding to group A of Weinreb and Shin in framework of spurious polycythemia.[40] The two patients with increased Epo levels were perhaps cases in a very early stage.

The better evaluation of PV by the bone marrow erythroid progenitor cultures in polycythemias in the study we report (+22%) leads us to reconsider the recommended diagnostic steps to follow in a suspected case of polycythemia. That investigation must occur at an earlier diagnostic step, at the same time that the TRCV and the arterial oxygen saturation are measured. Likewise, one must now consider the possibility of diagnosing PV at an early stage when the TRCV increase is still minimal. As early treatment and control improve the survival median of PV,[41] one may expect that early diagnosis will improve the outlook.

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REFERENCES

1. Berlin NI· Diagnosis and classification of the polycythemias. Semin Haematol 12:339, 1975

2. Wasserman LR: The management of polycythaemia vera Br J Haematol 21.371, 1971

3. Najean Y, Triebel F, Dresch C Pure erythrocytosis. Reappraisal of a study of 51 cases. Am J Hematol 10:129, 1981

4. Pearson TC, Wetherley-Mein G: The course and complications of idiopathic erythrocytosis. Clin Lab Haematol 1·189, 1979

5. Prchal JF, Axelrad AA. Bone marrow responses in polycythemia vera. N Engl J Med 290.1382, 1974

6. Anderson NF, Beckman B, Beltran G, Fischer JW, Stuckey WJ· Erythropoietin—independent erythroid colony formation in patients with erythroleukemia (M6) and related disorders. Br J Haematol 52:311, 1982

7. Villeval JL, Cramer PH, Lemoine F, Henri A, Bettaieb A, Bernaudin F, Beuzard Y, Berger R, Flandrin G, Breton-Gorius J, Vainchenker W: Early erythroblastic leukemias correspond to distinct stages of erythroid differentiation (submitted for publication).

8 Pennatur-Das R, Alpen E, Vichinsky E, Garcia J, Lubin B Evidence for the presence of CFU-E with increased in vitro sensitivity to erythropoietin in sickle cell anemia. Blood 63:1168, 1984

9. Eaves AC, Eaves CJ: Abnormalities in the erythroid progenitor compartments in patients with chronic myelogenous leukemia (CML). Exp Hematol 7·65, 1979 (suppl 5)

10. Eridani S, Batten E, Sawyer B· Erythroid colony formation in primary thrombocythaemia· Evidence of hypersensitivity to erythropoietin Br J Haematol 55:157, 1983

11. Lutton JD, Levere RD: Endogenous erythroid colony formation by peripheral blood mononuclear cells from patients with myelofibrosis and polycythemia vera Acta Haematol 62:94, 1979

12. Eaves CJ, Eaves AC. Erythropoietin (Ep) dose response curves for three classes of erythroid progenitors in normal human marrow and in patients with polycythemia vera. Blood 52.1196, 1978

13. Lacombe C, Casadevall N, Varet B: Polycythemia vera: In vitro studies of circulating erythroid progenitors. Br J Haematol 44:189, 1980

14 Zanzani ED, Lutton JD, Hoffman R, Wasserman LR: Erythroid colony formation by polycythemia vera bone marrow in vitro dependence on erythropoietin. J Clin Invest 59·841, 1977

15. Brubaker LH, Wasserman LR, Goldberg JD, Pisciotta AV, McIntyre OR, Kaplan ME, Modan B, Flannery J, Harp R: Increased prevalence of polycythemia vera in parents of patients on Polycythemia Vera Study Group protocols. Am J Hematol 16.367, 1984

16. Manoharan A, Garson OM: Familial polycythemia vera: A study of 3 sisters. Scand J Haematol 17:10, 1976

17. International Committee for Standardization in Haematology (CSH): Standard techniques for the measurement of red cell and plasma volumes. Br J Haematol 25:801, 1973

18. Rutenberg A, Rosales CI, Bennett JM: An improved histochemical method for the demonstration of leukocyte alkaline phosphatase activity: Clinical application. J Lab Clin Med 65:698, 1965

19. Lau KS, Gottlieb CW, Wasserman LR, Herbert V: Measurement of serum vitamin B12 level using radioisotope dilution and coated charcoal. Blood 26.202, 1963

20. Duhamel G: Histopathologie du Ganglion Lymphatique. Paris, Masson, 1979

21 Ellis JJ, Silver RT, Coleman M, Geller SA: The bone marrow in polycythemia vera. Semin Hematol 12:433, 1975

22. Stephenson JR, Axelrad AA. Quantitative assay method for erythropoietin "In vitro." Endocrinology 88:1519, 1971

23. Goldwasswer E, Eliason JF, Sikkema D: An assay for erythropoietin in vitro at the milliunit level. Endocrinology 97:315, 1975

24. Boffa GA, Gallien-Lartigue O, Tambourin P, Soulier JP. In vitro bioassay for minute erythropoietin quantities using foetal mouse liver cells. XVII Congress of the International Society of Hematology, Paris, 1978, p 678

25. Guilbert LJ, Iscove NN: Partial replacement of serum by selenite, transferrin, albumin and lecithin in hematopoietic cell cultures. Nature 262:594, 1976

26 McLeod DL, Shreeve MM, Axelrad AA: Improved plasma culture system for production of erythrocytic colonies in vitro: Quantitative assay method for CFU-E. Blood 44:517, 1974

27. Worton RG, McCulloch EA, Till JE: Physical separation of hemopoietic stem cells from cells forming colonies in culture. J Cell Physiol 74.171, 1969

28. Eaves AC, Eaves CJ: In vitro studies of erythropoiesis in polycythemia vera, in Dunn CDR (ed): Current Concept in Erythropoiesis. New York· Wiley, 1983, pp 167–187

29. Adamson JW, Fialkow PJ, Murphy S, Prchal JF, Steinmann L: Polycythemia vera· Stem cell and probable clonal origin of the disease. N Engl J Med 295.913, 1976

30. Clement S, Eberlin A, Najean Y, Chedeville A· Two different in vitro growth patterns for erythroid precursors in 18 patients with pure erythrocytosis. Scand J Haematol 29:319, 1982

31. Duhamel G, Najman A, Andre R: L'histologie de la moelle osseuse dans la maladie de Vaquez et le problème de la myélosclérose Nouv Rev Fr Hematol 10.209, 1970

32. Kurnick JE, Ward HP, Block MH: Bone marrow sections in the differential diagnosis of polycythemia Arch Pathol 94.489, 1972

33 Lundin PM, Ridell B, Weinfeld A: The significance of bone

marrow morphology for the diagnosis of polycythemia vera Scand J Haematol 9:271, 1972

34. Erslev AJ, Caro J, Kansu E, Miller O, Cobbs E: Plasma erythropoietin in polycythemia. Am J Med 66:243, 1979

35. Koeffler HP, Goldwasser E: Erythropoietin radio-immuno assay in evaluating patients with polycythemia. Ann Intern Med 94:44, 1981

36. De Klerk G, Rosengarten PCJ, Vet RJWM, Goudsmit R: Serum erythropoietin (ESF) titers in polycythemia. Blood 58:1171, 1981

37. Adamson JW, Fialkow PJ: Annotation. The pathogenesis of myeloproliferative syndromes. Br J Haematol 38:299, 1978

38. Croizat H, Amato D, McLeod DI, Eskinazi D, Axelrad AA: Differences among myeloproliferative disorders in the behavior of their restricted progenitor cells in culture. Blood 62.578, 1983

39. Ogawa M. Annotation. Cellular mechanisms of myeloproliferative disorders. Br J Haematol 58:563, 1984

40. Weinreb NJ, Shih CF: Spurious polycythemia. Semin Hematol 12:397, 1975

41 Hoffman R, Wasserman LR Natural history and management of polycythemia vera. Adv Intern Med 24:255, 1979

Enhanced Recovery of Gut-Associated Lymphoid Tissue by Infusion of Buffy Coat Cells and Gut Leukocytes in the Murine Syngeneic Bone Marrow Transplantation Model

By George S. Leventon, Sulabha S. Kulkarni, Robert A. White, and Axel R. Zander

Ways of accelerating recovery of the mucous membrane immune system in lethally irradiated mice following syngeneic bone marrow transplantation were studied over a 35-day period by quantification of jejunal intraepithelial lymphocytes (IELs) and lamina propria plasma cells. Recovery after a low bone marrow dose allowing 100% animal survival (LBM) was compared with a high (five times minimal) dose (HBM), or a minimal dose augmented with equal numbers of buffy coat cells (LBM + BC) or small gut mucosal lymphocytes (LBM + GL). The maximal decline and subsequent peak repopulation of IELs were: LBM, days 7 through 14, peaking suboptimally by day 28; HBM, day 14, peaking suboptimally but higher than LBM by day 35; LBM + BC, days 11 through 14, peaking at control levels by day 35; and LBM + GL, day 7, peaking at control levels by day 28. The IEL decline was most severe with LBM and HBM treatment and least with LBM + GL. All transplant groups experienced maximal plasma cell decline by day 7. LBM had the most severe depletion, and LBM + GL had the least. Recovery to control levels for the LBM, HBM, LBM + BC, and LBM + GL groups occurred by days 28, 21, 21, and 14, respectively. In all instances, >95% of the plasma cells were IgA positive.

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SEPSIS, a major risk of bone marrow transplantation, is caused by pathological organisms that often enter the blood stream through mucosal portals.[1,2] These sites are protected by the "local" or mucosal immune system, which is distinguished from the systemic immune system by a number of characteristics. These differences include the optimal route of antigen presentation,[3–8] the preferential homing of mucosal lymphoid cells back to mucosal sites,[9–14] and secretory IgA (S-IgA) as the major immunoglobulin product.[15–16] Gut-associated lymphoid tissue (GALT) consists of Peyer's patches and mesenteric lymph nodes, lamina propria lymphocytes and plasma cells, and intraepithelial lymphocytes (IELs). A migration pattern of lymphoid cells occurs between these tissues, with cells going from the Peyer's patches to mesenteric lymph nodes and then to the thoracic duct lymph and the general circulatory system, with subsequent seeding into the lamina propria and mucosal epithelium.[10,13]

Zander and colleagues[17] examined syngeneic bone marrow engraftment in the mouse (BDF 1) model, demonstrating more rapid engraftment when the quantity of the marrow was increased fivefold over the 100% minimum survival bone marrow dose, or when buffy coat cells were included in equal numbers to the minimal 100% survival marrow innocula. The purpose of this study was to determine in the same model if engraftment of the mucosal membrane immune system could similarly be potentiated by increasing the bone marrow dose or by the addition of buffy coat cells or gut-associated lymphoid cells to the minimal 100% survival allowing bone marrow innocula. This was assessed by quantifying jejunal IELs and plasma cells and, in the latter case, by determining the antibody class.

MATERIALS AND METHODS

Animals and preparation for transplantation Conventionally reared 8-week-old male BDF 1 mice (Simonson Laboratory, Gilroy, Calif) fed ad lib standard laboratory chow and acidified water (pH 2.5) were used throughout these experiments. Animals were prepared for syngeneic transplantation or given lethal irradiation without bone marrow rescue by exposure to 900 rad ^{60}Co total body irradiation (TBI) from a single source at a source-target distance of 130 cm and a rate of 52 rad/min.

Preparation of cells for transplantation. Bone marrow was obtained by flushing femurs and dispersing cells in Hanks' balanced salt solution (HBSS) and removal of bony spicules by passage through a steel mesh. Blood for buffy coat cells was obtained from the retroorbital space Lymphocytes were obtained from the small bowel epithelium and lamina propria as described previously, in which surface antigen analysis demonstrated 36% of the cells to be null, 35% to be positive for Thy 1.2, and 29% to bear surface immunoglobulins [18] (The latter subpopulation indicates significant admixing of lamina propria lymphocytes along with the IELs recovered by this technique.) In brief, this was achieved as follows. The small bowel was removed and the chyme was eliminated by flushing with phosphate-buffered saline (PBS). The epithelial layer was digested by distending the lumen of the gut for 1 hour at 37 °C with 4.5 mL of a hyaluronidase-containing solution (4,700 USP U of bovine testis hyaluronidase (US Biochemical Corp, Cleveland, OH, 38593), and 100 U each of penicillin and streptomycin per milliliter in Eagle's minimum essential medium with vitamins, amino acids, 0.17 glucose, and 20% fetal bovine serum (FBS)). The resultant suspension was harvested by gently milking and flushing the cells out of the lumen with 2 to 3 mL of PBS. The lymphocytes were purified by layering over discontinuous Percoll (30%, 40%, and 70% in PBS with 5% FBS, 0.1% glucose, and 80 µg/mL crude DNase (Sigma Biochemical Corp, St Louis, D0876 [DN]) and centrifuging at 628 g (mid-gradient) at 0 °C to 4 °C for 30 minutes. The lymphocytes banded at the 40% to 70% interface.

Treatment groups and sampling points To obtain a uniform donor and recipient population, we performed experiment en masse with animals having the identical date of birth as well as identical shipping, housing, and feeding conditions. Donor cells were pooled to ensure uniformity of the infused bone marrow, buffy coat cells and musocal lymphocytes. Groups of 40 irradiated mice each were

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infused with 2.0×10^5 bone marrow cells (low bone marrow dose allowing 100% survival, LBM), 1.0×10^6 bone marrow cells (five times minimal dose, HBM), 2.0×10^5 bone marrow cells + 2.0×10^5 buffy coat cells (LBM + BC), or 2.0×10^5 bone marrow cells + 2.0×10^5 gut lymphocytes (LBM + GL) as a 0.5-mL aliquot in the tail vein. Viability of infused cells was determined by 0.4% trypan blue exclusion. Enumeration was done with a hemocytometer using phase-contrast microscopy. All dilutions were made in HBSS. Twenty animals received irradiation alone with sham transplantation of 0.5 mL HBSS (TBI). An untreated control group of six mice was examined Six animals from each treatment group were scored on day 7, 11, 14, 21, 28, and 35 postmanipulation.

Quantification of jejunal IEL and plasma cells, and plasma cell immunoglobulin determination. A 3.0-cm section of gut from the junction of the proximal one-third and distal two-thirds and the small bowel was removed and placed in 10% buffered formalin for 5 minutes until stiffened. It was then quartered into 0.75-mL cylinders and placed back in formalin to complete fixation All four samples from each animal were embedded on end 90 ° to the long axis of the bowel in a solitary paraffin block. Serial 2- to 3-μm cross-sections were prepared and stained with methyl green pyronin (highlighting RNA-containing material allowing for more rapid identification of plasma cells) and by the three-layer indirect peroxidase–antiperoxidase method[19] for IgA, IgM, or IgG.[19] The latter was done as follows. Dewaxed slides were immersed for 30 minutes in 0.5% H_2O_2 (in methanol) to destroy endogenous peroxidase activity, absorbed with 3% normal goat sera (Cappel Laboratories, Westchester, PA) for 5 minutes, and blotted. They were then reacted serially with primary antiserum for 30 minutes (1:100 rabbit anti-mouse IgG, IgA, or IgM, Litton Bionetics Inc, Charleston, SC, 8403-04, 8403-01, or 8403-09) with link antiserum for 5 minutes (1:20 goat anti rabbit IgA, US Biochemical 25427) and then peroxidase–antiperoxidase for 5 minutes (1:100 rabbit antiperoxidase complexed with horseradish peroxidase, US Biochemical 2464), which was visualized by a 5- to 6-minute exposure to diamino benzidine HCl (0.5% wt/wt in Tris-buffered saline pH 7.6 (TBS) with freshly added H_2O_2 to 0.5% vol/vol). All antibody dilutions and slide washings between antibody reactions were done with TBS. All reactions were done at room temperature in a humidifying chamber. The slides were counterstained by a 10-second dip in filtered Mayer's hematoxylin

IELs were quantified by the number of them contained within 500 epithelial cells divided by 5,[20] and plasma cells were quantified by the number per cross-section Each of six animals within a sampling point had four repeated measures scored.

Statistical treatment. The Student-Newman-Keuls *t* test was used to analyze data sets between different experimental treatments at the same time intervals (cross-sectional data) and within the same treatment at different time intervals (longitudinal data). The α level was set at 0.05. All comparisons were planned in advance.

RESULTS

All animals receiving bone marrow transplants survived through day 35, and all irradiated nontransplanted mice died by day 14. No evidence of gastrointestinal pathology other than radiation-induced lymphoid depletion and transient acute radiation damage to the epithelium was observed. The sloughing of neurotic cells and integrity of the epithelial lining of the gut was completely established by day 7.

IELs. After irradiation, the numbers of IELs dropped sharply (Table 1). Starting from a control value of 12.9, by day 7 the TBI, LBM, HBM, LBM + BC, and LBM + GL groups fell to 3.5, 2.0, 8.1, 6.9, and 8.2, respectively. The IEL counts of the LBM group did not fluctuate significantly from day 7 through day 14; by day 14, the IELs of the HBM group had reached their maximal decline (2.1) approaching this level. The low point for the LBM + BC group was also reached at day 14 (4.6); it was significantly higher than the day 14 LBM and HBM IEL counts. The nadir was reached by day 11 for LBM + GL-treated animals (7.6). The IEL levels of the TBI group remained stable through day 11 at 3.5. As indicated in Table 1, at day 21 IEL levels for the HBM, LBM + BC, and LBM + GL groups formed one set of statistically similar values, with their means ranging from 8.8 to 10.0. The untreated control group formed a second higher set (12.9), and the day 21 LBM group formed a third lower value set (4.3) ($P < .05$). The LBM group reached peak repopulation levels from day 28 to 35 with a mean of 7.8 to 8.2 IELs. The recovery of the HBM group peaked at day 21 to 35, with the mean ranging from 10.0 (day 21) to 11.1 (day 28) IELs. Both the LBM and HBM peaks fell short of the control level ($P < .05$) and were different from each other ($P < .05$). The maximum repopulation of IELs produced by the LBM + BC or LBM + GL regimens did not differ statistically from the control level, at day 35 (12.0) for the former and at day 28 (12.0) through 35 (12.0) for the latter treatment.

Plasma cells. As with the IEL levels, all animals exposed to TBI had a rapid drop in plasma cells by day 7

Table 1. IEL Levels

	Day					
Treatment	7	11	14	21	28	35
TBI	3.5 (3.2–3.8)	3.5 (3.3–3.8)	—	—	—	—
LBM	2.0 (1.5–2.6)	1.4 (0.9–1.8)	1.7 (1.4–2.1)	4.3 (3.5–5.2)	8.2 (7.4–9.0)	7.8 (7.1–8.4)
HBM	8.1 (7.0–9.2)	6.4 (5.8–7.1)	2.1 (1.6–2.6)	10.0 (8.9–11.1)	11.1 (10.2–12.0)	10.3 (9.1–11.6)
LBM + BC	6.9 (6.4–7.4)	5.0 (4.2–5.7)	4.6 (3.7–5.4)	9.2 (8.2–10.2)	10.1 (9.7–10.5)	12.0 (11.1–12.8)
LBM + GL	8.2 (7.7–8.8)	7.6 (6.9–8.3)	10.1 (9.0–11.2)	8.8 (7.6–9.9)	12.0 (11.2–12.7)	12.0 (11.5–12.4)

IELS jejunal intraepithelial lymphocytes. TBI, total body irradiation; LBM, low bone marrow dose allowing 100% animal survival; HBM, high (5x minimal) bone marrow dose, BC, buffy coat cells; GL, small gut mucosal lymphocytes

Untreated control 12.9 (12.3–13.5); mean (95% confidence interval for the mean).

regardless of whether they had received any of the transplantation regimens (Table 2). Numerically, there was a decline from a pretreatment mean of 365 to 71.7, 67.6, 93.6, 101, and 139 for the TBI, LBM, HBM, LBM + BC, and LBM + GL manipulations, respectively. There was no significant difference between the TBI and LBM groups or between the HBM and LBM + BC groups. By day 11, all the transplantation treatments produced a significant elevation in number of plasma cells for their respective day 7 levels ($P < .05$), whereas the TBI group continued to decline (45.6) ($P < .05$). Cell repopulation approached the control level by day 28, 21, 21, and 14 for the LBM (376), HBM (335), LBM + BC (347), and LBM + GL (310) treated mice.

Immunoglobulin class of plasma cells. The predominant immunoglobulin class found in the lamina propria plasma cells of the control group was IgA (>99%). IgM- and IgG-bearing plasma cells were rarely present, accounting for <1% of all plasma cells. This relationship was maintained with minor variations during recovery of the plasma cell population after all the transplantation treatments. On day 21, IgM-positive cells accounted for 2.4% of the LBM + BC and 2.2% of the plasma cells of the LBM + GL groups. Also on day 21, 1.5% of the plasma cells in the LBM + BC-treated animals contained IgG. In all instances, >95% of the plasma cells were IgA positive.

DISCUSSION

In this study, irradiation induced a rapid loss of plasma cells with a half-life of ~4 days. This is consistent with the 4.7-day half-life previously reported for mice.[21] Tritiated mouse peripheral lymph node plasma cells have been shown to persist as long as 65 days after lethal irradiation and bone marrow rescue.[22] IEL depletion was also rapid, but by day 7 it had stabilized at 27% of control levels, representing a large fraction of radioresistant cells. Syngeneic bone marrow transplantation was able to reverse plasma cell and IEL depletion in mice caused by irradiation with 900 rad ^{60}Co. Differences in the degree of initial loss, rates of recovery, and maximal repopulation levels demonstrated a positive dose response to bone marrow. Augmenting bone marrow at a ratio of 1:1 with buffy coat cells, and especially small gut mucosal lymphocytes proved beneficial in promoting morphologic recovery. This may represent a concentration gradient of cells destined for the mucosal immune system. This would not be an unexpected observation considering the previously mentioned migratory path followed by cells of the mucosal immune system. The shifts in IEL subpopulations that occurred or the functional defects that remained after normal plasma cell density and immunoglobulin class (IgA) were regained are still unknown.

IELs are more radioresistant than are classical lymphocytes.[23] Recently, in rat radiation chimeras conditioned by a single dose of 1,000 rad, it was reported that the IEL population 3 to 5 weeks postbone marrow transplantation was almost equally divided between donor and host cells, whereas few host peripheral lymph node lymphocytes remained.[24] These investigators demonstrated a bone marrow origin for the donor IELs. It is likely that the regenerated host IELs arose from the relatively large radioresistant gut IEL population seen in the present study. IELs are migratory and mitotically active. In our study, infusion of gut mucosal lymphocytes along with bone marrow markedly blunted the depopulation and hastened repopulation of IELs in the gut.

The biological roles of IELs have yet to be satisfactorily determined. Until recently, they were thought to have a large (35% to 100%) T cell component, as judged by their reaction to various antisera (including monoclonal agents) thought to be T cell specific,[10,18,25-33] yet the thymus has proved not to be necessary for their processing.[24,25] T cell markers are known to be also expressed on NK cells[34] and Thy-1–positive cells have been observed in appreciable numbers within the lymphoid tissues of nude mice.[35] It has been postulated that there may be classes of pre-T stem cells committed to specific organs by passing the thymus.[36] We wonder if it is wise to use the term "T cell" for these cells which bypass the thymus and may have biological properties unique from "classical" T cells. IELs have a large number of cells with overlapping Ly 1 and Ly 2 antigens[18,32] and, in accepting the current premises that this represents an ontogenically immature state, we previously suggested that the gut epithelium might be a site for IEL maturation.[18] IELs have been shown to be active in NK, K, and cytotoxic T cell killing.[33,37-39] They are in close proximity to enterocytes and the epithelial processes of lamina propria dendritic macrophages, which are constitutionally positive for Ia antigen.[40] What bearing this arrange-

Table 2. Plasma Cell Levels

Treatment	Day 7	11	14	21	28	35
TBI	71.7 (61.8–81.6)	45.6 (36.0–55.1)	—	—	—	—
LBM	67.6 (55.2–80.0)	163 (140–186)	197 (168–226)	260 (231–290)	376 (343–408)	327 (288–366)
HBM	93.6 (74.5–113)	175 (152–197)	172 (143–200)	335 (302–368)	363 (321–406)	303 (244–361)
LBM + BC	101 (87.5–115)	166 (144–189)	258 (227–289)	347 (318–376)	309 (277–341)	324 (289–360)
LBM + GL	139 (129–150)	191 (169–213)	310 (280–340)	331 (285–377)	321 (300–342)	298 (273–323)

Abbreviations as in Table 1.

Untreated control 365 (344–385); mean (95% confidence interval for the mean).

ment has on IEL function remains to be determined. A more rapid IEL engraftment may be beneficial in the case of autologous or syngeneic bone marrow transplant, but, if IELs are involved in the pathogenesis of graft *v* host response (GVHR), this could prove detrimental.

S-IgA has a primary antiseptic rather than aseptic role. Diminished secretory immunoglobulin levels need not be synonymous with overt GI disease[41,42] if good hygiene is maintained and trauma is avoided. Case reports, however, have associated a poorly functioning secretory immunoglobulin system with susceptibility to gut infection and malabsorption syndromes in humans, which were reversed on oral administration of secretory immunoglobulins.[43,44] S-IgA blocks antigen and microbial entry into the body by preventing adherence to the epithelia,[45–49] binding to antigens within the mucus meshwork,[50] stimulating goblet cells to release mucus,[51] and potentiating the action of lactoferrin.[52,53] Materials that have complexed with S-IgA that do gain access to the gut wall and the portal system are selectively taken up in the liver.[54]

In our study, there was rapid logarithmic loss of plasma cells with irradiation. This is consistent with the rapid loss of salivary S-IgA reported in a patient who had extra corporeal cesium irradiation of blood for management of chronic lymphocytic leukemia[55] but is at partial odds with the autopsy study of Beschorner and colleagues.[56] They reported gut lamina propria plasma cell densities in a semiquantitative manner, finding only a mild loss of cells following engraftment failure or failure of sustained engraftment in patients who survived >28 days. Those with graft *v* host disease (GVHD) had severe depletion, especially of IgA-bearing plasma cells. Unfortunately, antemortem secretory immunoglobulin levels were not obtained to correlate function with morphology.

Present studies on the fluctuations in gut plasma cells and IELs after lethal irradiation and syngeneic bone marrow transplantation in the mouse demonstrated that a severe loss of GALT occurs over a prolonged period of time. This loss can be abated and recovery potentiated by augmenting the transplant regimen with buffy coat cells or gut lymphoid cells. The fact that mucosal immunoincompetency may occur in patients after TBI and bone marrow transplantation may explain the high rate of associated mucosal infection in patients with allogeneic and autologous bone marrow transplants.[1,2] The addition of buffy coat cells to bone marrow grafts may have potential in enhancing the recovery of the GALT and thereby decreasing the risk of infections derived from the gastrointestinal tract.

REFERENCES

1. Dreizen S, McCredie KB, Dicke KA, Zander AR, Peters LJ: Oral complications of bone marrow transplantation in adults with acute leukemia. Postgrad Med 66:187, 1979

2. Winston DS, Gale RP, Meyer DV, Young LS· Infectious complications of human bone marrow transplantation. Medicine 58·1, 1979

3 Bull GG, Mc Kee CM: Respiratory immunity in rabbits. 7. Resistance to intranasal infection in absence of demonstrable antibodies. Am J Hygiene 9·490, 1929

4. Walsh TE, Cannon PR: Immunization of the respiratory tract A comparative study of the antibody content of respiratory and other tissues following active, passive regional immunization J Immunol 35:31, 1938

5. Ogra PL, Kazan DT: Distribution of poliovirus antibody in the serum, nasopharynx or alimentary tract following segmental immunization of lower alimentary tract with poliovirus. J Immunol 102:1423, 1969

6. Yamamoto F, Anacker RL, Ribi E: Macrophage migration inhibition studies with cells from mice vaccinated with cell walls of *Mycobacterium bovis* BCG: Relationship between inhibitory activity of lung cells and resistance to airborne challenge with *Mycobacterium tuberculosis* H37RV. Infect Immun 1:595, 1970

7. Barclay WR, Busey WM, Dalgard DW et al: Protection of monkeys against airborne tuberculosis by aerosol vaccination with *Bacillus* Calmette-Guerin. Am Rev Respir Dis 107:351, 1973

8. Pierce NF, Cray WC· Determinants of the colonization, magnitude and duration of a specific mucosal IgA plasma cell response in enterically immunized rats. J Immunol 128:1311, 1982

9. Craig SW, Cebra JJ: Peyer's patches: An enriched source of precursors for IgA producing immunocytes in the rabbit. J Exp Med 134:188, 1974

10. Guy-Grand D, Griscelli C, Vassalli P The gut associated lymphoid system: Nature and properties of large dividing cells Eur J Immunol 4.435, 1974

11. Craig SW, Cebra JJ Rabbit Peyer's patches, appendix and popliteal lymphnode B lymphocytes: A comparative analysis of the membrane immunoglobulin components and plasma cell precursor potential J Immunol 114.492, 1975

12. Rudzik R, Clancy RL, Perey DYE, Day RP, Bienenstock J: Repopulation with IgA containing cells of bronchial and intestinal lamina propria after transfer of homologous Peyer's patch and bronchial lymphocytes. J Immunol 114:1599, 1975

13. McDermott MR, Bienenstock J: Evidence for a common mucosal immunological system I. Migration of B immunoblasts into intestinal, respiratory and genital tissues. J Immunol 122.1892, 1979

14 Tseng J: Repopulation of the gut lamina propria with IgA containing cells by lymphoid cells isolated from the gut lamina propria Eur J Immunol 14.420, 1984

15 Hanson LA: Comparative immunological studies of the immune globulins of human milk and of blood serum. Int Arch Allergy 18:241, 1961

16 Tomasi TB, Tan EM, Soloman A, Pendergast RA: Characteristics of an immune system common to certain external secretions. J Exp Med 121·101, 1965

17. Zander AR, Verma DS, Spitzer G, Huynh L, Johnston D, Beran M, Dicke KA: Enhancement of hemopoietic recovery after bone marrow transplantation with the addition of nucleated blood cells in mice Blood 56:1132, 1980

18. Leventon GS, Kulkarni SS, Meistrich ML, Newland JR, Zander AR: Isolation of murine small bowel intraepithelial lymphocytes J Immunol Methods 63·35, 1983

19. Sternberger L: Immunocytochemistry (ed 2). New York, Wiley, 1979, p 104

20 Nakano A: Uber die zahl der wanderzellen in der darmepithelsehicht und die kollelution zwischen der wanderzellen zahl und dem lymphatischen apparatder darmwand und insbesondere den noduli lymphatici aggregati. Hokkaido Ig2 (Sapporo) 7.559, 1929; ref. Jpn J Med Sci Trans Anat 3:115, 1931 (abstr)

21. Mattioli CA, Tomasi TB: The life span of IgA plasma cells from the mouse intestine. J Exp Med 438:452, 1973

22. Miller JJ III, Cole LJ. Radiation resistance of long-lived

lymphocytes and plasma cells in mouse and rat lymph nodes. J Immunol 98.982, 1967

23. Fichtelius KE: Radiosensitivity of the lymphocytes within the gut epithelium Arch Pathol Microbiol Scand 75:27, 1969

24 Mayrhofer G, Whately RJ: Granular intraepithelial lymphocytes of the rat small intestine. I. Isolation, presence in T lymphocyte deficient rats and bone marrow origin. Int Arch Allergy Appl Immunol 71:317, 1983

25. Ropke C, Everett NB: Kinetics of intraepithelial lymphocytes in the small intestine of thymus-deprived mice and antigen deprived mice. Anat Rec 185:101, 1976

26. Guy-Grand D, Griscelli C, Vassalli P. The mouse gut T lymphocyte, a novel type of T cell. Nature, origin, and traffic in mice in normal and graft-versus-host conditions. J Exp Med 148:1661, 1978

27. Bartnik W, Remine S, Chiba M, Thayer W, and Shorter R: Isolation and characterization of colonic intraepithelial and lamina propria lymphocytes. Gastroenterology 78:976, 1980

28. Janossy G, Tidman N, Selby WS, Thomas JA, Granger S, Kung PC, Goldstein G· Human T-lymphocytes of inducer and suppressor type occupy different microenvironments. Nature 288:81, 1980

29. Davies MDJ, Parrott DMV: Preparation and purification of lymphocytes from the epithelium and lamina propria of murine small intestine. Gut 22:481, 1981

30 Selby WS, Janossy G, Goldstein G, Jewell DP: T lymphocyte subsets in human intestinal mucosa: The distribution and relationship to MHC-derived antigens Clin Exp Immunol 44:453, 1981

31. Selby WS, Janossy G, Jewell VP: Immunohistological characteristics of intraepithelial lymphocytes of human gastrointestinal tract. Gut 22:169, 1981

32. Guy-Grand D, Vassalli P Nature and function of gut granulated T-lymphocytes, in Strober W, Hanson LA, Sell KW (eds): Recent Advances in Mucosal Immunity. New York, Raven, 1982

33. Tagliabue A, Befus AD, Clark DA, Bienenstock J: Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. J Exp Med 155:1785, 1982

34. Herberman RB, Holden HT: Natural cell mediated immunity. Adv Cancer Res 27:305, 1978

35. Loor F, Roelants GE: High frequency of T lineage lymphocytes in nude mouse spleen. Nature 251.229, 1974

36. Volf D, Kosuta D, Kuqi S, Tonljenovic M, Zenku E: Organ-specific commitment of pre-T and pre-B stem cells Exp Hematol 13:336, 1985 (abstr)

37. Arnaud-Battandier F, Bundy BM, O'Neill M, Bienenstock J, Nelson D: Cytotoxic activities of gut mucosal lymphoid cells in guinea pigs. J Immunol 121 1059, 1978

38. Tagliabue A, Luini W, Soldateschi D, Boraschi D: Natural killer activity of gut mucosal lymphoid cells in mice. Eur J Immunol 11:919, 1981

39. Lyscom N, Brueton MJ: Intraepithelial lamina propria, and Peyer's patch lymphocytes of the rat small intestine: Isolation and characterization in terms of immunoglobulin markers and receptors for monoclonal antibodies. Immunology 45 775, 1982

40. Mayrhofer G, Pugh CW, Barclay AN· The distribution, ontogeny and origin in the rat of Ia positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine Eur J Immunol 13:112, 1983

41. Rosen FS, Janeway CA: The gammaglobulins. III. The antibody deficiency syndromes N Engl J Med 275:709, 1966

42. Brown WR, Savage DC, Dubois RS, Alp MH, Mallory A, Kern F· Intestinal microflora of immunoglobulin-deficient and normal human subjects Gastroenterology 62·1143, 1972

43. Krakauer, Zinneman HH, Hong R: Deficiency of secretory IgA and intestinal malabsorption. Am J Gastroenterol 64:319, 1975

44. Strober W, Krakauer R, Klaeveman HL, Reynolds HY, Nelson DL· Secretory component deficiency. A disorder of the IgA immune system N Engl J Med 294:351, 1976

45. Freter R: Mechanism of action of intestinal antibody in experimental cholera: II. Antibody-mediated antibacterial reaction at the mucosal surface Infect Immun 2·556, 1970

46. Gibbons RJ, Van Houte J. Selective bacterial adhesion to oral epithelial surfaces and its role as an ecological determinant. Infect Immun 3.567, 1971

47. Williams RC, Gibbons RJ: Inhibition of bacterial adherence by secretory immunoglobulin A: A mechanism of antigen disposal. Science 177.697, 1972

48 Fubara E, Freter R. Protection against enteric bacterial infection by secretory IgA antibodies. J Immunol 111:395, 1973

49. Stoliar OA, Pelley RP, Kaniecki-Green E, Klaus MH, Carpenter CCJ: Secretory IgA against enterotoxins in breast milk. Lancet 1 1258, 1976

50. Walker WA, Wu M, Isselbacher KJ, Bloch KJ: Intestinal uptake of macromolecules. III. Studies on the mechanism by which immunization interferes with antigen uptake. J Immunol 115.854, 1975

51. Walker WA, Wu M, Bloch KJ· Stimulation by immune complexes of mucus release from goblet cells of the rat small intestine. Science 197:370, 1977

52. Bullen JJ, Rogers HJ, Leign L: Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. Br Med J 1:69, 1972

53. Rogers HJ, Synge C: Bacteriostatic effect of human milk on *Escherichia coli:* The role of IgA. Immunology 34.19, 1978

54. Socken DJ, Simms ES, Nagy BR, Fisher MM, Underdown BJ: Secretory component-dependent hepatic transport of IgA antibody-antigen complexes. J Immunol 127.316, 1981

55. Friedman BK, Greenberg B, McNamara TF: Extracorporeal cesium irradiation on secretory and serum immunoglobulins. J Dent Res 54:96A, 1975 (abstr)

56. Beschorner WE, Yardley JH, Tutschka PJ, Santos GW: Deficiency of intestinal immunity with graft-vs-host disease in humans J Infect Dis 144:38, 1981

Studies on the Effect of Vanadate on Endocytosis and Shape Changes in Human Red Blood Cells and Ghosts

By S.L. Schrier, Irene Junga, and Lisa Ma

When amphipathic cationic drugs are added to intact human RBCs, the RBCs first undergo a stomatocytic shape change and then, if relatively large amounts of drug are added and if the metabolic state of the RBC is appropriate, endocytic vacuoles form. Vanadate has a structural similarity to the transition state of phosphate, which presumably accounts for its ability to inhibit phosphohydrolases, although other actions of vanadate have been described. Vanadate inhibited three forms of drug-induced endocytosis in intact RBCs despite the fact that the three drugs chosen (primaquine, chlorpromazine, and vinblastine) are known to have differing requirements for RBC ATP. Vanadate also inhibited the stomatocytic shape change produced by primaquine, chlorpromazine, and vinblastine, but not the stomatocytosis produced by low pH. Vanadate had no effect on RBC echinocytosis produced by lysophosphatidylcholine. In studying endocytosis in hypotonic, leaky, "white" ghosts, we discovered that vanadate inhibited only the endocytosis produced by Mg-ATP and not the endocytosis produced by manipulations that directly attack the cytoskeletal proteins. These findings suggest that ATP hydrolysis has a role in some forms of amphipathic cation-induced stomatocytosis and endocytosis in intact RBCs. In addition, studies in ghosts support the idea that Mg-ATP does indeed produce "energized" endocytosis dependent on utilization or hydrolysis of ATP.

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WHEN AMPHIPATHIC anions and cations are added to RBCs in vitro, they produce, respectively, echinocytosis and stomatocytosis.[1] Somewhat similar shape alterations also occur under certain circumstances in vivo.[2] Stomatocytosis can be extended in vitro to proceed to the formation of endocytic vacuoles, which are inside-out portions of the RBC plasma membrane that form preferentially in the invaginated portion of the cup.[3] The irreversible loss of membrane surface area into endocytic vacuoles reduces the surface area/volume ratio[3] and results in the formation of a spherostomatocyte. Continuation of the process results in RBC lysis.[3,4] A role of RBC metabolism in this in vitro process was deduced from the observation that glucose deprivation could prevent primaquine-induced hemolysis.[4] Some forms of drug-induced endocytosis (ie, that produced by chlorpromazine) can occur in ATP-depleted RBCs, an observation that tended to uncouple the relationship between RBC metabolism and drug-induced RBC shape change.[5] Subsequently, it was noted that most forms of drug-induced stomatocytosis and endocytosis in vitro take place more readily in glucose-supplemented, ATP-replete RBCs.[6] It has been suggested that ATP has an important but poorly understood role in controlling intact RBC shape.[7] On the basis of experiments performed with ghosts, a shape-mediating role of Mg-ATP[8] acting through the membrane Mg^{2+}-ATPase has been proposed.[9]

These observations led us to suspect that there is a role for ATP and perhaps ATP hydrolysis in drug-induced stomatocytosis and the subsequent endocytosis in intact RBCs. An opportunity for evaluating the potential role of ATP hydrolysis in RBC stomatocytosis and endocytosis was provided by recent observations on the properties of the several forms of vanadium. Ionic vanadium, under certain circumstances, has a structural similarity to the transition state of phosphate, thereby perhaps accounting for its ability to inhibit phosphohydrolases at micromolar concentrations.[10,11] Vanadate inhibition of RBC membrane Na^+, K^+-ATPase[12] and Ca^{2+}, Mg^{2+}-ATPase[13,14] has been reported, whereas incubation of vanadate with intact RBCs has inhibited the sodium pump in a manner consistent with inhibition of the pump-related RBC membrane Na^+, K^+-ATPase.[12] Therefore, we tested a hypothesis that ATP hydrolysis was important in drug-induced stomatocytosis and endocytosis in intact human RBCs by studying the effect of vanadate on RBC shape change and endocytosis. In addition to inhibiting the phosphohydrolases, vanadate can inhibit phosphatases, stimulate adenyl cyclase[15] and, at very high concentrations, can produce RBC metabolic alterations[16,17] and even cause shape changes.[17] Therefore, experiments using vanadate must be carefully controlled and interpreted.

Use of vanadate also offered us the opportunity of testing a hypothesis on endocytosis produced in white ghosts.[18,19] We and others had identified two forms of endocytosis in white ghosts. One form, called "nonenergized" endocytosis, is produced by hypotonicity, EDTA, or trypsin, all of which appear to act by stripping away the cytoskeleton.[19] The other form is produced by addition of Mg-ATP and has been called "energized endocytosis,"[18] although the mechanism of energization has not been identified. If vanadate blocked Mg-ATP-induced energized endocytosis, it would mean that ATP hydrolysis was probably required.

MATERIALS AND METHODS

All reagents used were reagent grade or better Sodium orthovanadate was obtained from Fisher Scientific Company, and its concentration was verified.[20] The solutions were adjusted to pH 7.5, and only solutions that were clear and devoid of color were used.[21] Carrier-free ^{32}Pi was obtained from New England Nuclear Corp (Boston). Lysophosphatidylcholine was obtained from Sigma Co, St Louis. Trypsin was obtained from the Worthington Chemical Co,

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Freehold, NJ. RBC membrane actin was purified as previously described.[22] Silicon 550 Fluid was obtained from Dow Corning. [G-^{3}H] Vinblastine sulfate-$^{-3}$H 11.4 Ci/mmol was obtained from Amersham International, Amersham, England. Chlorpromazine HCl (benzene ring-^{3}H) 39.1 Ci/mmol was obtained from New England Nuclear. Protosol and Biofluor were also obtained from New England Nuclear.

Freshly drawn, heparinized venous blood was obtained from normal human donors according to a protocol approved by the Stanford University Committee on Human Experimentation. The blood was centrifuged at room temperature, and the plasma and buffy coat were removed, after which the RBCs were washed once with 15- to 20-fold volumes of 0.15 mol/L of NaCl and then three times with either cold Hanks' solution or phosphate-buffered saline (PBS) pH 7.4 containing 2 mg glucose/mL.

Ghost preparation. White ghosts were made exactly as previously described, using three washes of 5 mmol/L of phosphate buffer pH 8.0 at a ratio of 40 vol of lysis solution to 1 vol of packed RBCs.[22] The protein concentration was determined by the method of Lowry, using five times recrystalized bovine serum albumin (BSA) as standard.

ATPase assays. The several different ATPase activities displayed by ghosts were measured exactly as previously described using γ ^{32}P-ATP as substrate[22] The ^{32}Pi generated as a result of ATPase action was quantitatively recovered in the isobutanol-benzene extract.[23] Four ATPases were measured. All assays contained ~100 μg of membrane protein, 2.5 mmol/L of Mg^{2+}, and 1.5 mmol/L of γ-^{32}P-ATP, which defined the Mg^{2+}-ATPase. For the Na^+, K^+-ATPase, 30 mmol/L of K^+ and 100 mmol/L of Na^+ were added For the Ca^{2+}, Mg^{2+}-ATPase, 0.12 mmol/L of Ca^{2+} was added. For the actin-activated ATPase, 100 μg of RBC membrane actin was added to 100 μg of RBC membrane protein.

RBC endocytosis Endocytosis was induced in intact RBCs by adding primaquine, chlorpromazine, and vinblastine as described previously.[3,6,24] To test the effect of vanadate on RBC endocytosis, it was preincubated with RBCs suspended in plasma or Hanks' solution for 30 to 60 minutes at 37 °C to allow passage of vanadate into the RBC interior.[12,15] At the end of the preincubation period, the endocytosis-inducing drug was added, and the timing of the incubation was begun and continued at 37 °C for the length of time indicated in the text and Tables 1 through 6 The extent of endocytosis was monitored by phase microscopy and by the radioisotopic method, which is based on the trapping of ^{57}Co-vitamin B_{12} within endocytic vacuoles.[3,6,24] Preliminary experiments indicated that vanadate, at the concentrations used (5 to 100 μmol/L), produced neither RBC shape changes nor endocytosis, nor did it interfere with the binding of the ^{57}Co-vitamin B_{12}–transcobolamin complex to the RBC surface. ATP levels were measured by the coupled hexokinase-G6PD assay[25] in intact RBCs before and after incubation with the endocytosis-inducing drugs alone or in combination with vanadate. It was important to determine whether the inhibitory effects of vanadate were reversible. Therefore, RBCs as described above were incubated without and with vanadate at 37 °C. Then, relying on the published information of vanadate efflux,[15] we washed the RBCs twice with a 20-fold volume of Tris-buffered saline pH 7.2 containing 1% BSA. We then incubated them overnight at 37 °C at a hematocrit of 10 in Hanks' solution containing 10% autologous plasma, 5 mmol/L of glucose, and penicillin 200 U/mL and streptomycin 200 μg/mL In the morning, the RBCs were washed again three times with Hanks' solution and were then studied as above for their capacity to undergo cationic amphipath-induced RBC shape change and endocytosis.

RBC shape changes. To produce echinocytosis, intact washed RBCs were incubated in PBS-glucose (2 mg/mL) at 2% hematocrit with 6 μg/mL of lysophosphatidylcholine for up to 30 minutes at 37 °C without or with the addition of vanadate. Samples of 20 μL were removed at given time intervals from 5 to 30 minutes, added to 200 μL of 1% glutaraldehyde in PBS, and then examined by phase microscopy. The proportion of RBCs of different morphologies was recorded using the nomenclature and classification of Bessis.[2] The effect of vanadate on shape change reversibility was then measured by removing the suspending media and washing the RBC pellet serially with either 1% BSA in PBS, or 1% BSA plus 30 μmol/L of vanadate in PBS. Morphologic assessment by phase microscopy was made of the RBCs after each wash Selected samples were also photographed by Nomarski interference microscopy. The effect of vanadate on induction of stomatocytosis was also studied. The amphipathic cations, primaquine, chlorpromazine, and vinblastine, were used to produce stomatocytosis; the incubations were carried out as described for endocytosis. Acid-pH was also reported by Deuticke[1] to induce stomatocytosis. Therefore, washed intact RBCs at a hematocrit of 2% were incubated in PBS pH 7.4 as a control, and then with both 0.1 mol/L of PO_4 buffer pH 5.2 or citrate buffer 0.1 mol/L pH 5 2 at 37 °C for 30 minutes without or with concurrent addition of 30 μmol/L of vanadate. Samples were obtained for morphologic analysis. Reversal of shape change was then performed by washing either with 1% BSA in PBS pH 7.4 or 1% BSA plus 30 μmol/L of vanadate in PBS pH 7 4.

Drug entry into RBCs. The effect of vanadate on the uptake of chlorpromazine and vinblastine was measured by a method previously used, with a single modification[26] Instead of diluting and washing the RBC pellet after incubation, we layered the samples on top of a 1-ml silicon barrier in a 1.5-mL Eppendorf centrifuge tube and immediately centrifuged them at room temperature for 2 minutes at full speed. The clear supernatant formed a distinctly visible band above the barrier, and the RBC pellet was densely packed at the bottom of the tube. The top supernatant fraction was carefully removed and added to a scintillation vial. The RBC pellet was harvested by cutting off the tip of the tube and placing it in a scintillation vial. One milliliter of Protosol/ethanol (1:2) was added to all vials, which were digested at 60 °C for 90 minutes and then allowed to cool, after which 0.3 mL of 30% H_2O_2 was added and the samples were stored overnight at room temperature. Ten milliliters of Biofluor was added, and the vials were shaken; then 0.5 mL of 0.5 N HCl was added. The mixture was shaken, allowed to cool for 2 hours, and then counted by liquid scintillation spectrometry in a Beckman LS 230 counter The specific activity of endocytosis-inducing drugs was adjusted by adding radioisotopes to "cold" drugs to provide a stock standard solution. The chlorpromazine specific activity used was 1.13×10^6 cpm/μmol, and the vinblastine specific activity was 3.46×10^5 cpm/μmol.

Ghost endocytosis. Endocytosis in white ghosts was induced by three methods.[19] Addition of 20 ng/mL of trypsin or 0.1 mmol/L of EDTA produces endocytic vacuoles presumably by stripping off segments of the cytoskeletal proteins, ie, nonenergized endocytosis. Conversely, addition of 3 mmol/L of Mg-ATP to leaky ghosts suspended in 50 mmol/L of TES pH 7.5 produces energized endocytosis.[18] The extent of endocytosis was measured semiquantitatively by observing coded ghosts samples under phase microscopy and quantitatively by the acetylcholinesterase method originally described by Jarrett and Penniston[27] In all reported experiments, the semiquantitative morphologic assessment was in excellent agreement with the results of the acetylcholinesterase assay Vanadate had no effect on the acetylcholinesterase assay (data not shown). To determine if the effect of vanadate was reversible, ghosts incubated without and with vanadate were washed three times in 50 mmol/L of TES and then restudied for their capacity to undergo Mg-ATP–induced endocytosis.

Radiophosphorylation studies of ghosts during endocytosis In attempts to explore the molecular mechanism by which Mg-ATP might cause ghost endocytosis, RBCs were radiolabeled by incubating them under sterile conditions for 20 hours with 100 to 500 μCi of ^{32}P/mL packed RBCs[22] Ghosts were then prepared and incubated as above with 50 mmol/L of TES buffer, and 3.0 mmol/L of Mg-ATP without and with the addition of 30 μmol/L of vanadate to block endocytosis. Following incubation at 37 °C for a time sufficient to cause extensive endocytosis in the samples free of vanadate, ghosts were separated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels using the Laemmli system as previously described[22] The gels were analyzed by densitometry, bands were cut out and weighed, and the proportion of total proteins was recorded. The extent of radiophosphorylation of the membrane proteins was then assessed semiquantitatively by ^{32}P-radioautography and quantitively by slicing out the relevant bands, dissolving them in 600 μL of 30% H_2O_2, adding aquasol, and measuring the radioactivity by liquid scintillation spectrometry From the radioisotopic data and protein concentrations, the ^{32}P specific activity of the individual phosphorylated proteins could be calculated.

RESULTS

ATPase assays. The ATPase assays were carried out in duplicate as described in the Materials and Methods section, and the samples were incubated 60 min at 37 °C both without vanadate and with the addition of 2 and 20 μmol/L of vanadate at the start of the incubation period. The results (Table 1) showed that all the ATPases were strongly inhibited by vanadate at 20 μmol/L and that Mg^{2+}-ATPase showed the greatest sensitivity, being inhibited 53% at 2 μmol/L of vanadate.

Ghost endocytosis and the effect of vanadate. Experiments were designed to test the effect of vanadate on energized endocytosis as produced by addition of 3 mmol/L of Mg-ATP, or on nonenergized endocytosis produced by incubation of ghosts with 20 ng/mL of trypsin or 0.1 mmol/L of EDTA. Incubation was carried out (as described in the Materials and Methods section) at 37 °C, with samples observed at 0, 15, 30, 45, and 60 minutes. In each case, a sample with buffer alone and another with an appropriate concentration of vanadate used were incubated in parallel. Acetylcholinesterase results were correlated with independent coded morphologic observations. Vanadate in concentrations of 30 μmol/L had no inhibitory effect on endocytosis produced by either EDTA or trypsin. In contrast, it profoundly inhibited Mg-ATP endocytosis (Table 2A). This effect of vanadate was completely reversible. Ghosts incubated with 30 μmol/L of vanadate were washed three times with 50 mmol/L of TES, after which their ability to undergo Mg-ATP–induced endocytosis was unimpaired as compared with values of ghost suspensions incubated in parallel but without prior vanadate addition (Table 2B). Ghosts were then prepared from RBCs that had been radiophosphorylated. Aliquots were taken for analyses at 0, 60, and 150 minutes of incubation at 37 °C. No differences in the amount and distribution of the membrane proteins were detectable by densitometry; nor were there detectable differences in the phosphorylation patterns of the membrane proteins among four samples containing: buffer only; 30 μmol/L of vanadate; 3 mmol/L of Mg-ATP, and 3 mmol/L of Mg-ATP plus 30 μmol/L of vanadate (data not shown).

Table 1. Effect of Vanadate on RBC Membrane ATPases

Vanadate Concentration (μmol/L)	Mg^{2+}-ATPase	Na^+, K^+-ATPase	Ca^{2+}, Mg^{2+}-ATPase	AA-ATPase
2.0	53 ± 17 (3)	26 (1)	12 ± 13 (3)	19 ± 7 (3)
20.0	75 ± 0.6 (3)	77 (1)	51 ± 14 (2)	69 ± 6 (4)

Number of experiments in parentheses. Values are expressed as percent of inhibition of the control value, mean ± SD.

Table 2. Effect of Vanadate on Endocytosis in Ghosts

Endocytosis Inducer	No of Experiments	Time of incubation at 37 °C	
		30 Min	60 Min
A			
Mg-ATP	5	97 ± 6	95 ± 7
Trypsin	7	0	0
EDTA	3	0	0
B			
Reversal of vanadate inhibition			
Mg-ATP	2	0	0

Results are expressed as percentage of inhibition ±SD produced by 30 μmol/L of vanadate.

Effect of vanadate on drug-induced endocytosis. Addition of up to 100 μmol/L of vanadate and incubation for up to 60 minutes at 37 °C had no effect on RBC shape or on the binding of the indicator ^{57}Co-vitamin B_{12}–transcobalamin complex to the RBC outer surface (data not shown). Vanadate was allowed to enter RBCs during a 60-minute preincubation at 37 °C. Endocytosis produced by all three drugs (Table 3) was inhibited by vanadate. The magnitude of the vanadate effect can be appreciated from a single experiment, in which primaquine endocytosis trapped 1,150 cpm of $^{57}Co/10^{10}$ RBCs but preincubation with 30 μmol/L of vanadate reduced that to 250 cpm/10^{10} RBCs. In the same experiment, control values for chlorpromazine endocytosis were 881 cpm/10^{10} RBCs, falling to 313 cpm/10^{10} RBCs on addition of 30 μmol/L of vanadate. Parallel values for

Table 3. Vanadate Inhibition of Drug-Induced Endocytosis in RBCs

Drug	No. of Experiments	Concentration of Vanadate (μmol/L)	Inhibition (%) of Endocytosis Mean ±SD
Primaquine			
3 mmol/L	4		
		7.5	53 ± 21
		15.0	75 ± 19
		30.0	95 ± 11
Chlorpromazine			
1 mmol/L	6		
		7.5	36 ± 29
		15.0	64 ± 27
		30.0	90 ± 17
Vinblastine			
0.6 mmol/L	6		
		7.5	10 ± 15
		15.0	29 ± 26
		30.0	77 ± 18

vinblastine were 1,781, falling to 619. Phase microscopy revealed that >50% of RBCs had one or more vacuoles in control cationic amphipath-treated samples, whereas after vanadate <10% of the RBCs contained vacuoles except in the vinblastine sample, in which ~20% of the RBCs contained vacuoles. Primaquine endocytosis seemed most susceptible, with an I_{50} of ~7.5 μmol/L of vanadate.

Because it is known that reduction in ATP levels can impair drug-induced endocytosis, particularly primaquine endocytosis,[6] six experiments were performed with six different donors to measure ATP levels in RBCs exposed to endocytosis-inducing drugs without or with the addition of vanadate. Table 4 shows the results of a single experiment and indicates that no change in ATP levels occurred as a consequence of incubation with amphipathic cations or vanadate.

Effect of vanadate on RBC shape change. Because our experimental design called for coded morphologic assessment of RBC shapes during drug-induced endocytosis, it became quickly apparent that vanadate not only blocked endocytosis but also blocked the acquisition of the spherostomatocytic shape change that invariably precedes endocytosis (Fig 1). Figure 1 shows the results with primaquine; however, vinblastine and chlorpromazine stomatocytosis were similarly blocked. Primaquine and vinblastine produce the stomatocytic shape change over a period of minutes, whereas the chlorpromazine shape change is virtually instantaneous. Nevertheless, preincubation with vanadate blocked the chlorpromazine-induced stomatocytosis (data not shown) as effectively as it blocked primaquine and vinblastine stomatocytosis. Higher concentrations of primaquine (4 to 6 mmol/L) could partially overcome the shape inhibition of 30 μmol/L of vanadate (data not shown) but not of 100 μmol/L of vanadate.

The next question was whether vanadate could block other forms of shape change. Acid pH produces stomatocytes I and II.[1,2] We therefore incubated RBCs as described in the Materials and Methods section without or with vanadate in isotonic citrate or phosphate buffer at pH 5.2. At intervals, RBCs were removed, fixed with glutaraldehyde, and examined under phase microscopy. In two such experiments, vanadate had no effect on the acquisition of the stomatocytic shape produced by acidic buffers (data not shown). The shape change produced by acid pH was not readily reversed by four 60-fold volume washes in 1% BSA dissolved in PBS pH 7.4. Therefore, the role of vanadate on the reversal of the acid pH stomatocytic shape change could not be assessed.

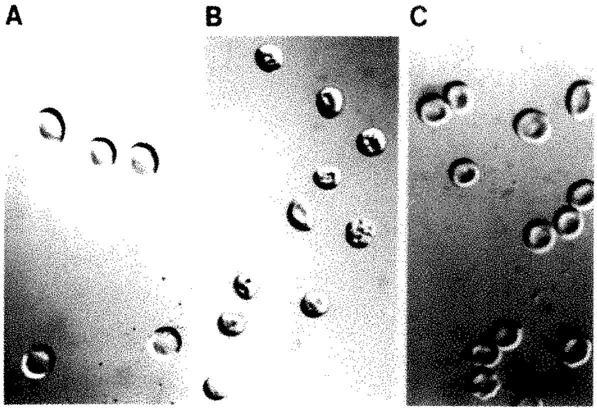

Fig 1. Nomarski interference microscopy, original magnification 900x; current magnification 432x. (A) Control RBCs incubated without primaquine or vanadate for 45 minutes at 37 °C. The addition of 100 μmol/L of vanadate during a 30-minute preincubation at 37 °C resulted in the same discocytic picture. (B) RBCs were incubated with 4 mmol/L of primaquine for 45 minutes at 37 °C. Note the extensive and uniform conversion to spherostomatocytes I.[2] (C) RBCs were preincubated with 100 μmol/L of vanadate for 30 minutes at 37 °C before 4 mmol/L of primaquine was added; the reaction was then continued for an additional 45 minutes at 37 °C. Note the persistence of the discocytic shape in essentially all RBCs.

Echinocytosis was induced as described in the Materials and Methods section by adding 6 μg/mL of lysophosphatidylcholine, which produces echinocytes II and III. Addition of 30 μmol/L of vanadate had no effect on the appearance or extent of this shape change. The reversibility of this shape change was then studied by washing RBCs in 1% BSA in PBS; neither preincubation with vanadate nor addition of vanadate to the wash solution interfered with the prompt reversal of the echinocytic shape change to the discocytic shape.

Reversibility of vanadate effect on RBC stomatocytosis and endocytosis. To determine if the effect of vanadate was reversible, RBCs that had been pretreated with 30 μmol/L of vanadate were extensively washed and then incubated overnight under conditions calculated to enhance vanadate efflux. The results of one of two such experiments indicated (Table 5) that the effect of vanadate on endocytosis was reversible. The effects of vanadate on inhibition of stomatocytosis were also completely reversible (data not shown).

Effect of vanadate on drug entry into RBCs. It was possible that micromolar amounts of vanadate could block uptake of amphipathic drug into RBCs. Therefore, the effect of vanadate on drug uptake was measured using identical

Table 4. RBC ATP Levels

	Value
Initial RBC ATP value	1.2
Following incubation with	
Vanadate 30 μmol/L and:	
No drug added	1.3
Primaquine 3 mmol/L	1.3
Chlorpromazine 1 mmol/L	1.9
Vinblastine 0.6 mmol/L	1.6

Values are μmol ATP/mL packed RBCs.

Table 5. Reversibility of Vanadate Inhibition of RBC Endocytosis

Drug	Fresh RBCs Treated With Vanadate 30 μmol/L (%)	Vanadate-Treated (30 μmol/L) RBCs, Washed and Incubated Overnight (%)
Primaquine		
3 mmol/L	76	5
Chlorpromazine		
1 mmol/L	31	0

Values are percentage of inhibition.

Table 6. Effect of Vanadate on Drug Uptake Into RBCs

Drug Concentration	Time of Incubation at 37 °C (min)	Vanadate Addition	Concentration of Drug in RBCs*	Concentration of Drug in Supernatant†	Recovery of Drug (%)
Vinblastine 0.5 mmol/L	45	0	0.52	0.39	83
		30 μmol/L	0.51	0.38	83
Chlorpromazine 1.0 mmol/L	15	0	0.76	0.79	79
		30 μmol/L	0.71	0.84	81

*μmol/mL of packed RBCs.
†μmol/mL of supernatant.

concentrations and ratios of drug, RBCs, buffer, and plasma, and the same time intervals we used to study endocytosis. Tritiated radioisotopes of vinblastine and chlorpromazine are available and we have previously studied their uptake into RBCs. The recoveries of radioisotopic drug were in the order of 80%. Vanadate produced no difference in RBC uptake of either chlorpromazine or vinblastine. One of the two experiments performed is shown in Table 6.

DISCUSSION

The chemistry of vanadium[28] and the oxyvanadium compounds[15,28] has been the object of considerable study because of their potential physiological role. At nanomolar to micromolar concentrations and at physiologic pH, the predominant species is pentavalent metavanadate anion (VO_3^-).[15,28] The metavanadate may resemble the transition state of phosphate, thus allowing it to insert into phosphate subsites of some ATP binding sites. Perhaps it is by this mechanism that metavanadate inhibits the RBC membrane Na^+, K^+-ATPase,[11,12] and Ca^{2+}, Mg^{2+}-ATPase,[13,14] as well as various phosphohydrolases in kidney, muscle, nerve, and liver.[15] These studies generally have been carried out on disrupted cell fractions. Vanadate can also inhibit the Na^+-K^+ pump when added to intact RBCs, but somewhat higher concentrations are required.[12,29] One explanation is that vanadate transverses the RBC membrane as the metavanadate anion; once inside the RBCs, however, substantial amounts of metavanadate are reduced to the tetravalent vanadyl cation (VO^{2+}),[15,21,29] a form that is less inhibitory to the Na^+, K^+-ATPase and that may also bind to cellular constituents.[29] In addition to its activity against ATPases, vanadate can also inhibit phosphatases, stimulate adenyl cyclase, and inhibit ATP-dependent proteases.[15,30] Of importance to our study is the fact that vanadate at the micromolar concentrations used (2 to 100 μmol/L) has no effect on RBC shape, deformability, osmotic fragility, or metabolism,[16,17] whereas it can affect all these modalities at considerably higher concentrations.

We initially confirmed reports that micromolar concentrations of vanadate inhibited four human RBC membrane ATPases: Mg^{2+}-ATPase, Na^+, K^+-ATPase, Ca^{2+}, Mg^{2+}-ATPase, and actin-activated ATPase (Table 1). The membrane preparation used for these measurements is essentially the same as the leaky hypotonic ghosts used to study endocytosis.

In ghosts suspended in 50 mmol/L of TES, endocytosis can be produced by 3 mmol/L of Mg-ATP (energized endocytosis)[18] and by attack on the cytoskeleton produced by trypsin or EDTA (nonenergized endocytosis).[19] Vanadate blocked only Mg-ATP endocytosis (Table 2), thereby suggesting that hydrolysis of ATP is the important factor in energized endocytosis. Fairbanks and colleagues[9] noted that vanadate blocked the discocytic shape transformation produced by isotonically sealing Mg-ATP within ghosts and suggested that vanadate inhibited Mg^{2+}-ATPase, which could be a shape-change–mediating enzyme. The evidence linking Mg^{2+}-ATPase to ghost shape change is based on a parallelism between the concentration of vanadate that inhibits Mg^{2+}-ATPase and the concentration of vanadate that inhibits Mg-ATP–induced ghost discocytosis.[9] Using a similar form of analysis, we suggested that in a somewhat different preparation—resealed red ghosts—endocytosis requires the Ca^{2+}-induced activation of Ca^{2+}, Mg^{2+}-ATPase, and that in the absence of Ca^{2+} or in the presence of inhibitors of Ca^{2+}, Mg^{2+}-ATPase red ghost endocytosis was inhibited.[31] Hayashi and Penniston used a series of alkylating agents and noted a strong correlation between inhibition of ATPase and inhibition of endocytosis in ghosts. They proposed that inhibition at a single site accounted for both actions.[32] In trying to determine which of the ATPases might be involved, they noted concurrent inhibition of ghost endocytosis and a low-affinity Ca^{2+}-stimulated Mg^{2+}-ATPase.[33] It is unlikely, however, that there is any free Ca^{2+} in these thoroughly washed white ghosts. Thus, the Mg^{2+}-ATPase as well as the actin-activated ATPase are candidates for the mechanisms by which Mg-ATP could energize, and vanadate block, ghost endocytosis. The prompt reversibility of the vanadate effect is consistent with the reversibility of vanadate inhibition of RBC membrane ATPases[15] and against the idea that vanadate caused the oxidation of sulfhydryl groups.[28] Attempts to identify a membrane protein dephosphorylated during Mg-ATP–induced endocytosis were unsuccessful. The methods used, however, were suitable only to detect dephosphorylation that might have occurred in β spectrin, protein 2.1, 3, or 4.1, but not the RBC membrane ATPases. In addition to ATPase action, the hydrolysis of Mg-ATP in ghost endocytosis may be produced by protein kinases, by enzymes involved in lipid phosphorylation[34] of ghost membranes, or by as yet unknown mechanisms.[35]

To study endocytosis in RBCs, a very different system than leaky ghosts, we preincubated intact RBCs with vanadate at concentrations reported to inhibit the Na^+-K^+ pump[12] and presumably the pump-related Na^+, K^+-ATPase; we observed a dose-dependent inhibition of drug-induced endocytosis (Table 3). It is highly unlikely that the RBC membrane Na^+, K^+-ATPase was involved in drug-induced endocytosis since neither ouabain addition[3] nor substitution of choline for Na^+ or K^{+24} have an effect on such endocytosis. We have previously shown that primaquine-induced endocytosis is absolutely dependent on active glycolysis and maintenance of ATP levels, whereas vinblastine and chlorpromazine endocytosis are reduced ~60% but not obliterated by RBC ATP depletion.[6] Preincubation of RBCs with vanadate did not reduce RBC ATP levels (Table 4). Because ATP was not depleted and micromolar additions of vanadate inhibited endocytosis, it is highly likely that it is not ATP in itself, but ATP hydrolysis that is involved in primaquine endocytosis to a great degree and in chlorpromazine and vinblastine endocytosis to a lesser extent.

While we were monitoring endocytosis morphologically, it quickly became apparent that the stomatocytosis (Fig 1) that invariably precedes the endocytosis was blocked by vanadate. We confirmed the fact that vanadate at the concentrations used (2 to 100 μmol/L) did not cause echinocytosis.[17] Therefore, its action in blocking the stomatocytic shape change was not to produce a conflicting or neutralizing shape alteration. We then addressed the question of whether vanadate blocked all forms of stomatocytosis or only those produced by cationic amphipaths. The stomatocytosis produced by pH 5 buffers was not inhibited by vanadate. The next question was whether vanadate would inhibit all forms of amphipath-induced shape change. Vanadate did not inhibit the echinocytosis produced by incubation of intact RBCs with lysophosphatydlcholine.

The use of vanadate indicates that all forms of stomatocytosis do not proceed by identical mechanisms because acid pH stomatocytosis is not blocked by vanadate but cationic amphipath-induced stomatocytosis is. The initial description of the bilayer couple hypothesis suggested that amphipath-produced stomatocytosis and echinocytosis were, in a sense, mirror images, with the charge of the amphipath passively determining its localization in the lipid bilayer and thus the site of membrane expansion.[36] Our studies show, however, that micromolar amounts of vanadate (metavanadate and vanadyl)[28] block the RBC stomatocytic shape change produced by cationic amphipaths but not lysophosphatydlcholine-induced echinocytosis. A possible explanation for this difference comes from recent data indicating that spin-labeled phospholipids may assume their transbilayer assymetric distribution in resealed RBC ghosts by a mechanism using Mg-ATP and blocked by 1 to 2 μmol/L of vanadate.[37] In intact RBCs, Mg-ATP is required to translocate aminophospholipids to the inner half of the bilayer.[38] Thus, vanadate could block Mg-ATP–mediated translocation of cationic amphipaths to the inner half of the bilayer. Against this proposal are the data in Table 6 indicating that vanadate does not inhibit the passage of such drugs through the membrane, during which time they would have access to the inner half of the bilayer. Mg-ATP–induced translocation of drug would only be involved if such drugs passed through the membrane in sealed channels and if their subsequent access from cytosol to the inner half of the bilayer was blocked by the cytoskeleton.

These studies indicate that it is not the presence of ATP but ATP hydrolysis, reversibly blocked by vanadate, that is required for certain forms of stomatocytosis and endocytosis in RBCs and endocytosis in ghosts. It is not proven that RBC and ghost endocytosis proceed by identical mechanisms, but the vanadate inhibition of both, at similar concentrations, suggests that the two are related. The pathways by which the hydrolysis of ATP leads to these morphologic changes must be determined.

REFERENCES

1. Deuticke B· Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. Biochim Biophys Acta 163·494, 1968

2. Bessis M. Red cell shapes. An illustrated classification and its rationale. Nouv Rev Fr Hematol 12:721, 1972

3. Ben-Bassat I, Bensch KG, Schrier SL Drug-induced erythrocyte membrane internalization J Clin Invest 51 1833, 1972

4. George JN, O'Brien RL, Pollack S, Crosby WH: Studies of in vitro primaquine hemolysis: Substrate requirement for erythrocyte membrane damage. J Clin Invest 45:1280, 1966

5. Feo C, Mohandas N: Clarification of role of ATP in red cell morphology and function. Nature 265·166, 1977

6 Schrier SL, Junga I, Krueger J, Johnson M: Requirements of drug-induced endocytosis by intact human erythrocytes. Blood Cells 4:339, 1978

7. Nakao M, Nakao T, Yamazoe S: Adenosine triphosphate and maintenance of shape of the human red cells Nature 187:946, 1960

8. Sheetz MP, Singer SJ: On the mechanism of ATP-induced shape changes in human erythrocyte membranes. J Cell Biol 73:638, 1977

9. Fairbanks G, Patel VP, Dino JE, Carter DP. Vanadate inhibition of the MgATP-dependent shape change of human erythrocyte ghosts J Cell Biol 95:254a, 1982 (abstr)

10. Macara IG Vanadium—an element in search of a role Trends Biochem Sci 5 92, 1980

11. Norby JG: Ligand interactions with the substrate site of Na, K-ATPase· nucleotides, vanadate, and phosphorylation Curr Topics Membrane Transport 19:281, 1983

12. Cantley LC Jr, Resh MD, Guidotti G. Vanadate inhibits the red cell (Na^+, K^+) ATPase from the cytoplasmic side. Nature 272:552, 1978

13. Rossi JPFC, Garrahan PJ, Rega AF. Vanadate inhibition of active Ca^{2+} transport across human red cell membranes. Biochim Biophys Acta 648:145, 1981

14. Bond GH, Hudgins PM: Inhibition of red cell Ca^{2+}-ATPase by vanadate. Biochim Biophys Acta 600:781, 1980

15. Heinz A, Rubinson KA, Grantham JJ: The transport and accumulation of oxyvanadium compounds in human erythrocytes in vitro. J Lab Clin Med 100:593, 1982

16. Ninfali P, Accorsi A, Fazi A, Palma F, Fornaini G. Vanadate affects glucose metabolism of human erythrocytes. Arch Biochem Biophys 226:441, 1983

17 Vives-Corrons JL, Jou JM, Ester A, Ibars M, Carreras J, Bartrons R, Climent F, Grisola S Vanadate increases oxygen affinity and affects enzyme activities and membrane properties of erythrocytes. Biochem Biophys Res Commun 103 111, 1981

18. Penniston JT. Endocytosis by erythrocyte ghosts; dependence upon ATP hydrolysis. Arch Biochim Biophys 153·410, 1972

19 Hardy B, Schrier SL The role of spectrin in erythrocyte ghosts endocytosis. Biochem Biophys Res Commun 81:1153, 1978

20. Goodno CC· Inhibition of myosin ATPase by vanadate ion Proc Natl Acad Sci USA 76 2620, 1979

21 Macara L, Kustin K, Cantley Jr LC: Glutathione reduces cytoplasmic vanadate: Mechanism and physiological implications. Biochim Biophys Acta 629.95, 1980

22. Schrier SL, Hardy B, Junga I, Ma L· Actin-activated ATPase in human red cell membranes Blood 58:953, 1981

23. Schrier SL, Giberman E, Danon D, Katchalski E: Studies on ATPase in sheared micro vesicles of human erythrocyte membranes. Biochim Biophys Acta 196 263, 1970

24. Schrier SL, Junga I, Seeger M: The mechanism of drug-induced erythrocyte vacuole formation. J Lab Clin Med 83.215, 1974

25 Beutler E: Red Cell Metabolism: A Manual of Biochemical Methods (ed 2). Orlando, Fla, Grune & Stratton, 1975, p 10

26 Schrier SL, Junga I: Entry and distribution of chlorpromazine and vinblastine into human erythrocytes during endocytosis. Proc Soc Exp Biol Med 168:159, 1981

27 Jarrett HW, Penniston JT: A new assay for endocytosis in erythrocyte ghosts based on loss of acetylcholinesterase activity. Biochim Biophys Acta 448:314, 1976

28. Rubinson KA: Concerning the form of biochemically active vanadium. Proc R Soc Lond 212:65, 1981

29. Cantley LC Jr, Aisen P: The fate of cytoplasmic vanadium. J Biol Chem 254 1781, 1979

30. Tanaka K, Waxman L, Goldberg AL: Vanadate inhibits the ATP-dependent degradation of proteins in reticulocytes without affecting ubiquitin conjugation. J Biol Chem 259:2803, 1984

31. Schrier SL, Bensch KG, Johnson M, Junga I: Energized endocytosis in human erythrocyte ghosts. J Clin Invest 56:8, 1975

32. Hayashi H, Penniston JT: Energy-dependent endocytosis in erythrocyte ghosts. Arch Biochem Biophys 159:563, 1973

33. Jarrett HW, Reid TB, Penniston JT: Concurrent inhibition of the low-affinity Ca^{2+}-stimulated ATPase and MgATP-dependent endocytosis in erythrocyte ghosts by naphthylmaleimide and carboncylcyanide-*m*-chlorophenylhydrazone. Arch Biochem Biophys 183:498, 1977

34 Fairbanks G, Patel VP, Dino JE: Biochemistry of ATP-dependent red cell membrane shape change. Scand J Clin Lab Invest 41:139, 1981

35 Jinbu Y, Nakao M, Otsuka M, Sato S: Two steps in ATP-dependent shape change of human erythrocyte ghosts. Biochem Biophys Res Commun 112:384, 1983

36. Sheetz M, Singer SJ: Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc Natl Acad Sci USA 71:4457, 1974

37. Seigneuret M, Devaux PF: ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: Relation to shape change Proc Natl Acad Sci USA 81 3751, 1984

38. Daleke DL, Huestis WH: Incorporation and translocation of aminophospholipids in human erythrocytes. Biochemistry 24:5406, 1985

Mechanism of Human Platelet Activation by Endotoxic Glycolipid-Bearing Mutant Re595 of *Salmonella minnesota*

By Sheila Timmons, Huzoor-Akbar, Jadwiga Grabarek, Marek Kloczewiak, and Jacek Hawiger

The mechanism through which human blood platelets interact with gram-negative bacteria with well-defined structural variations in endotoxic lipopolysaccharide was studied. Secretion of ^{14}C-serotonin and aggregation of platelets separated from plasma proteins were observed on challenge with rough mutant Re595 of *Salmonella minnesota* possessing a glycolipid outer layer composed of Lipid A and 2-keto-3-deoxyoctonate (KDO) but lacking heptose phosphate in the core and *O*-polysaccharide in its outer portion. Both ^{14}C-serotonin secretion and platelet aggregation were concentration-dependent, with a half-maximum response at the ratio of one bacterial colony-forming unit (CFU) to two platelets. The aggregation of human platelets induced by mutant Re595 was divalent cation-dependent and required secretion of ADP and fibrinogen from platelet storage granules because it was inhibited by chelators, by the ADP-splitting enzyme apyrase, and by monospecific antifibrinogen Fab fragments. The synthetic peptide analog of the platelet receptor recognition site on the γ chain of fibrinogen, γ 400–411, inhibited platelet aggregation induced by mutant Re595 (IC_{50} 160 μmol/L), whereas serotonin secretion was unaffected. Tetrapeptide, RGDS, analogous to human fibrinogen α chain (α 572–575) and to the cell adhesion site of fibronectin, also inhibited aggregation induced by mutant Re595 (IC_{50} 60 μmol/L). Secretion of ^{14}C-serotonin was preceded by a very rapid phosphorylation of a platelet protein of mol wt 47,000, which is associated with protein kinase C activation. Myosin light chain (mol wt 20,000) was also phosphorylated. Both phosphoproteins were dephosphorylated while secretion was reaching maximum. Furthermore, release of ^{3}H-arachidonic acid from platelet phospholipids and generation of thromboxane B_2 via the cyclooxygenase pathway were observed. Inhibition of this pathway with acetylsalicylic acid (10^{-4} mol/L) or indomethacin (5×10^{-4} mol/L) reduced ^{14}C-serotonin secretion and platelet aggregation. The role of Lipid A in the interaction of mutant Re595 with human platelets was deduced from the inhibitory effect of the Lipid A-binding protein present in *Limulus* amebocyte lysate. Likewise, polymyxin B, known to complex with Lipid A, was inhibitory. The reactivity of mutant Re595 toward platelets was attenuated by mild acid hydrolysis, during which KDO was dissociated from the glycolipid, and by alkaline hydrolysis, which breaks ester-linked fatty acids in Lipid A. In contrast to mutant Re595, strain S218 of *S minnesota* bearing "complete" endotoxic lipopolysaccharide did not induce secretion and aggregation of human platelets. The oligosaccharide fraction obtained from this strain inhibited platelet secretion of serotonin and aggregation caused by mutant Re595. These results indicate that an intact Lipid A-KDO structure in the rough mutant Re595 participates in the interaction with the platelet membrane and triggers more than one pathway of platelet activation.

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GRAM-NEGATIVE bacteria have many cellular targets, including blood platelets, granulocytes, monocytes, lymphocytes, erythrocytes, and endothelial cells.[1-4] Some of the effects of gram-negative bacteria contribute to the development of intravascular coagulation and septic shock, and it is estimated that ~71,000 patients per year in the United States develop gram-negative bacteremia, resulting in 18,000 deaths.[5] Bacteremia due to endotoxin-producing gram-negative rods is frequently complicated by thrombocytopenia.[6,7] Therefore, several attempts have been undertaken during the past years to establish in vitro evidence that human platelets interact with isolated endotoxic lipopolysaccharide. In contrast to rabbit platelets, which uniformly respond in vitro to isolated bacterial endotoxin,[8] human platelets have shown varying responses. Although studies have shown that secretion of serotonin from human platelets was induced by endotoxic lipopolysaccharide extracted by Boivin's method[9] and copper salt-treated endotoxin caused aggregtion of human platelets,[10,11] other studies were unable to demonstrate an interaction between isolated endotoxin and human platelets.[12] Most of the previous experiments with human platelets used endotoxic lipopolysaccharides extracted from gram-negative bacteria. These cell-free preparations may have different properties than the "native" form of cell wall-bound endotoxic lipopolysaccharide. Moreover, it is unclear which part of endotoxic lipopolysaccharide is responsible for the interaction of gram-negative bacteria with human platelets.

Endotoxic lipopolysaccharide of *Salmonella* and other gram-negative bacteria is composed of complex polysaccharide subunits covalently linked to Lipid A.[13] The polysaccharide is typically composed of two distinct parts, the core and the chains of *O*-polysaccharides. The core contains an unusual sugar, 2-keto-3-deoxyoctonate (KDO),[1] in addition to *N*-acetylglucosamine, glucose, galactose, heptose phosphate, and ethanolamine.[14] Whereas *O*-polysaccharide subunits

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determine the immunologic specificity of endotoxin, Lipid A appears to be responsible for many of its biologic effects, most notably, pyrogenicity, lethal toxicity, mitogenic stimulation of lymphocytes, macrophage activation, Hageman factor and complement activation, and induction of *Limulus* amebocyte lysate gelation.[2,13,15]

The rough mutant Re595 of *S minnesota* is known to possess a glycolipid composed of Lipid A and KDO but to lack core oligosaccharides and *O*-polysaccharide side chains due to a deficiency of specific enzymes present in smooth organisms.[14] The smooth strains of gram-negative bacteria undergo spontaneous transformation into polysaccharide-deficient rough variants in culture, especially when antibodies directed against somatic *O* antigens are present in the medium.[16] A Lipid A-like substance reappears in the circulation 24 hours after injection of endotoxin into baboons, and significant amounts of material cross-reacting with Lipid A-antiserum appear in the urine.[13] An endotoxin-like material has been detected by the *Limulus* amebocyte lysate assay in the serum of patients with gram-negative bacterial urinary tract infections whose blood cultures were negative.[17] It is likely that these measurements detected bacterial cell wall fragments bearing Lipid A-containing material which may also be reactive with platelets. In our study, the whole cells of rough mutant Re595 were compared with the smooth organism, strain S218. These bacteria, with a well-defined endotoxin composition, allowed us to establish the structural requirements for endotoxin's interaction with human platelets and the pathways of activation of human platelets by a cell wall-associated glycolipid composed of a Lipid A–KDO complex.

MATERIALS AND METHODS

Preparation of human platelets separated from plasma proteins for secretion and aggregation studies Blood was obtained from healthy volunteers who had not taken aspirin or any other medication for the preceding 8 days. Platelet-rich plasma was loaded with ^{14}C-serotonin (0.1 μCi/2mL, New England Nuclear, Boston) and platelets, labeled with ^{14}C-serotonin, were then separated from free amine and from plasma by combined albumin gradient centrifugation and Sepharose 2B gel filtration using modified Tyrode's buffer without phosphate and calcium, pH 7.35.[18] Secretion of serotonin was measured in the presence of imipramine (1.5 μg/mL) with continuous stirring at 900 rpm at 37 °C according to previously described procedures,[19] except that the reaction was stopped by chilling the platelets and adding 0.3% *p*-formaldehyde and 2 mmol/L of EDTA.[20]

Aggregation studies were done at 37 °C with stirring according to the method of Born,[21] using a Payton Dual-Channel Aggregometer (Payton Association, Buffalo). The aggregometer baseline (10%) was set using Tyrode's buffer and bacterial suspension as described previously.[19] Aggregation was measured using percentage of maximum transmission (T_{max}) and slope value which represented the change in 1 minute along a tangent line to the steepest increase in light transmission [18]

Preparation of anti-human fibrinogen Fab fragments. The antiserum prepared from rabbits immunized with human fibrinogen (Kabi, Sweden) was passed through a fibrinogen-Sepharose 4B affinity column as previously described.[22] The Fab fragments were prepared according to the procedure of Porter[23] and purified as previously described.[22]

Preparation of synthetic peptides Synthetic peptide analogs of the platelet receptor recognition site on human fibrinogen γ chain, NH_2-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Asp-Val-COOH (γ 400–411), and of the cell adhesion site on fibronectin and the α chain of human fibrinogen, NH_2-Arg-Gly-Asp-Ser-COOH (α 572–575)[24] were prepared as previously described.[25] Acetylation of the synthetic peptide γ 400–411 was done as described elsewhere.[25]

Phosphorylation of human platelet proteins Phosphorylation of human platelet proteins was studied according to the method of Lyons and co-workers[26] with the following modifications. Human platelets, separated from plasma proteins as above, were incubated with $^{32}PO_4$ (50 μCi/mL) for 60 minutes at room temperature without stirring After removal of free $^{32}PO_4$ by Sepharose 2B gel filtration, the platelets were challenged with bacteria for various incubation times at 37 °C with stirring. Control samples were incubated with buffer. The incubation mixture was immediately placed in an ice bath to stop the reaction and spun down in a microcentrifuge Platelet pellets were solubilized in 20 μL of 5% sodium dodecyl sulfate (SDS) in 0.15 mol/L of NaCl and an equal volume of Laemmli solubilizing solution[27] and 2-mercaptoethanol (final concentration 10%). Samples were boiled for 2 minutes and applied to SDS-containing polyacrylamide gradient (5% to 15%) slab gels for electrophoresis according to the method of Laemmli.[27] Molecular weight markers (BioRad, Richmond, Calif) were run simultaneously. The gels were stained with 0.25% Coomassie brilliant blue R, 45% methanol in 10% acetic acid, and subsequently destained using 7% methanol in 10% acetic acid The gels were dried on BioRad filter paper, exposed to Kodak (Rochester, NY) X-Omat-AR film using a Cronex lighting plus intensifying screen (Dupont, Wilmington, Del), and developed in a Kodak RPX-Omat processor. The autoradiographs were scanned at 600 nm using Response spectrophotometer (Gilford, Oberlin, Ohio) equipped with gel scan accessories and software that allowed quantitation of the integrated area under the peak.[28]

Measurement of release of ^{14}C-arachidonic acid from platelet phospholipids. Release of arachidonic acid from platelet phospholipids was measured according to the method described by Bills and colleagues [29] Twenty milliliters of platelets separated from plasma proteins, prepared as described above, were incubated at 37 °C for 2 minutes, and 60 μL (0 3 μCi) of ^{14}C-arachidonic acid was added. The sample was incubated at 37 °C for 1 hour without stirring The reaction was stopped with EDTA, and unincorporated ^{14}C-arachidonic acid was removed by gel filtration. One-milliliter aliquots of ^{14}C-arachidonic acid-labeled platelets were incubated, either with 0.5 U thrombin or 200 μg of freeze-dried mutant Re595 of *S minnesota* or smooth strain S218 for 5 minutes with continuous stirring Control samples were incubated with buffer. After incubation, 2.67 mL of EDTA (0 1 mol/L) was added to each tube, and subsamples were taken for radioactivity count. The radiolabeled phospholipids were extracted and chromatographed on a column (1 × 17 cm) containing 0.5 g of activated silicic acid on a glass wool plug.[29] Release of arachidonic acid from platelet phospholipids was calculated as the difference between the radioactivity counts in control and test samples of platelet phospholipids.

Measurement of metabolites of ^{3}H-arachidonic acid in human platelets by high-pressure liquid chromatography (HPLC). After platelets were labeled with ^{3}H-arachidonic acid and separated from plasma proteins, they were incubated with *S minnesota* Re595 or with thrombin and analyzed for free arachidonic acid and its metabolites such as cyclooxygenase products (thromboxane B_2, PGE_2, PGD_2, and $PGF_{2\alpha}$), 12-*L*-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-*L*-hydroxy-5,8,10,14 eicosatetranoic acid (HETE) by HPLC according to the procedure of Russell and Deykin.[30] Results were expressed as ^{3}H-arachidonic acid-derived radioactivity or as percentage of total radioactivity in the sample.

Measurement of lactate dehydrogenase leakage from human platelets. Lactate dehydrogenase (E C 1 1 1 27) was measured according to Bergmeyer and Bernt.[31] Results are expressed as percentage of total enzyme activity present in a platelet suspension lysed with 0.1% Triton X-100 (Sigma, St Louis)

Preparation of bacteria. The S218 strain of *S minnesota* and its rough mutant Re595 (kindly provided by Dr G Schmidt, Max-Planck Institute for Immunobiology, Freiburg-Zahringer, FRG) were grown overnight in heart infusion broth. The cultures were centrifuged at 1,200 g for 15 minutes, the supernatant was discarded, and the bacteria were suspended in saline. The bacteria were incubated in a 60 °C water bath for 30 minutes. These heat-killed bacteria were centrifuged at 1,200 g for 15 minutes, and the pellet was washed twice with saline and once with distilled water Finally, bacteria were freeze-dried; fresh suspensions were prepared on the day of experiment. The viable count was determined in the bacterial suspension before it was heated at 60 °C, and 1 mg of final freeze-dried bacterial preparations contained an equivalent of 10^{10} colony-forming units (CFUs) of strain S218 and 3×10^9 CFUs of mutant Re595.

Hydrolysis and measurement of KDO associated with mutant Re595. Dry bacteria (50 mg, mutant Re595) were washed twice with 7 mL of 0 17 N acetic acid and suspended in 7 mL of 0.17 N acetic acid. The suspension was heated to 100 °C in a boiling water bath for various times, up to 90 minutes.[32] After centrifugation, the supernatant and the pellet were collected, the pellet was washed twice with distilled water, and then both the supernatant and the pellet were freeze-dried. The thiobarbituric acid method[33] was used to measure KDO in the reconstituted supernatant and pellet.

Limulus gelation assay for endotoxic lipopolysaccharide. Cell wall endotoxic lipopolysaccharide in suspensions of *S minnesota* was measured by a modified method of Levin.[34] The bacterial suspensions were diluted in pyrogen-free water to obtain a concentration of 5 μg/mL and then mixed 1·10 (vol/vol) with *Limulus* amebocyte lysate (0.5 EU/mL). Pyrogen-free water mixed 1:10 with *Limulus* amebocyte lysate was used as a reference The reaction was measured in a Gilford Response spectrophotometer at the wavelength of 360 nm at room temperature for 60 minutes. The rate of reaction was measured as a slope value representing the change in absorbance in 1 minute by drawing a tangent line to the steepest part of the absorbance tracing

Modifications of Lipid A in mutant Re595 of S minnesota Alkaline hydrolysis of ester-linked fatty acids of Lipid A of mutant Re595 was done according to the method of Beckman.[35] In brief, bacterial cells (20 mg/mL) were suspended in water to which an equal volume of 0.2 N NaOH was added. The suspension was incubated at 37 °C for 30 minutes and centrifuged at 1,200 g for 10 minutes, and the pellet was washed twice with distilled water and lyophilized. Treatment of mutant Re595 with polymyxin B was done according to the method of Morrison and Jacobs[36] Polymyxin B sulfate, 15 mg, sterile powder (Burroughs Wellcome, Research Triangle Park, NC), was added to 2 mg *S minnesota* Re595 suspended in 1 mL of H_2O, incubated at room temperature for 30 minutes, and dialyzed against H_2O for 2 hours with frequent changes of H_2O Neutralization of mutant Re595 with *Limulus* amebocyte lysate (Pyrotell, 0 5 EU U/mL, Cape Cod Associates, Woods Hole, Mass) was done by mixing 50 μL of suspension of mutant Re595 (2 mg/mL) in Tyrode's buffer, with 50 μL of *Limulus* amebocyte lysate for 10 minutes at room temperature without stirring. The bacteria were then washed once with Tyrode's buffer, suspended in 50 μL of Tyrode's buffer, and then added to platelets in the aggregometer

Reagents. Acetylsalicylic acid (aspirin) and indomethacin USP were obtained from Merck, Sharp, and Dohme (West Point, Pa); hirudin from Sigma (St Louis); bovine thrombin (topical) from Parke Davis (Morris Plains, NJ), ^{14}C-serotonin, ^{14}C-arachidonic acid, and ^{3}H-arachidonic acid were purchased from New England Nuclear; and Limulus Amebocyte Lysate (Pyrotell, 0.5 EU/mL) from Cape Cod Associates. Apyrase (grade I, Sigma) was purified and assayed according to the procedure of Traverso-Cori and co-workers.[37]

RESULTS

Activation of human platelets induced by rough mutant Re595 but not by the smooth strain S218 of S minnesota.

On addition of gram-negative bacteria to a suspension of human platelets, which were free of plasma proteins, two patterns of interaction were observed. Rough mutant Re595 induced aggregation and secretion of ^{14}C-serotonin, which reached 55% at 10 minutes. When the smooth strain S218 was added, no response from platelets was observed (Fig 1). Platelet aggregation and secretion of ^{14}C-serotonin induced by mutant Re595 were concentration-dependent. The effective concentration of mutant Re595 that induced a half-maximal response (EC_{50}) was 25 μg/mL, which roughly corresponds to one bacterial CFU per two platelets (Fig 2). Smooth strain S218 remained without measurable effect over a wide range of concentrations. Secretion of serotonin induced by mutant Re595 was not accompanied by lysis of platelets because lactate dehydrogenase, a cytoplasmic marker, was not significantly increased at the end of the incubation period (9.2% ± 1.5% *v* control 4.8% ± 0.8%).

Role of platelet endogenous ADP and fibrinogen in aggregation induced by mutant Re595. The aggregating effect of mutant Re595 on human platelets separated from plasma

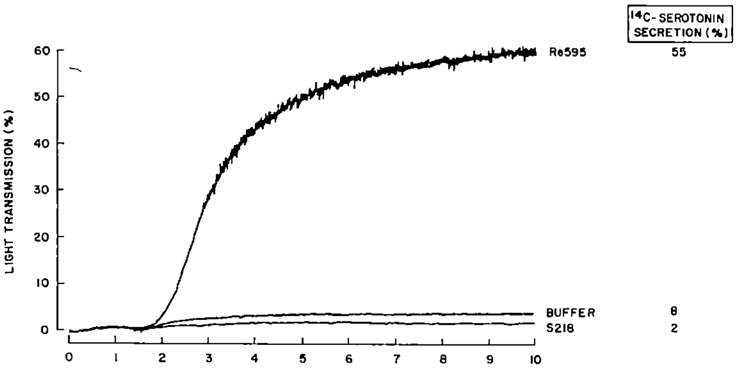

Fig 1 Time course of aggregation of human platelets challenged with mutant Re595 of *Salmonella minnesota* as compared with strain S218 and control buffer without bacteria. Bacteria (100 μg/mL) or buffer were added at time zero to platelet suspension (2 × 10^8/mL) free of plasma proteins. After 10-minute incubation at 37 °C with stirring, secretion of ^{14}C-serotonin was measured as described in the Materials and Methods section.

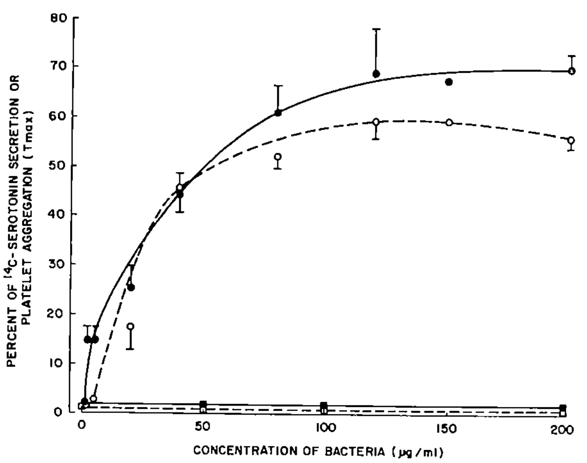

Fig 2. Concentration-dependent induction of ^{14}C-serotonin secretion (●) and platelet aggregation (○) by mutant Re595 of *Salmonella minnesota* as compared with smooth strain S218 (■, □). Bars represent ± SEM of three experiments.

proteins raised the question of whether this process was dependent on endogenous ADP, stored in platelet dense granules and secreted along with serotonin. Addition of the ADP-splitting enzyme apyrase inhibited aggregation of platelets in a concentration-dependent manner, thus indicating the role of secreted ADP in platelet aggregation (Fig 3).

The participation of platelet fibrinogen secreted from α-granules in aggregation of platelets induced by Re595 was examined using anti-human fibrinogen Fab antibody fragments. Monovalent Fab fragments were previously shown to inhibit thrombin-induced aggregation of human platelets separated from plasma proteins.[38] Anti-fibrinogen Fab caused concentration-dependent inhibition of platelet aggregation. At 40 μmol/L, Fab fragments inhibited platelet aggregation by 60% whereas secretion of ^{14}C-serotonin remained unaffected (90% of control).

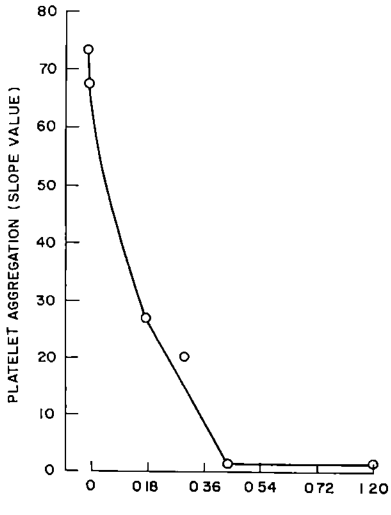

Fig 3. Apyrase inhibition of platelet aggregation induced by mutant Re595. Purified apyrase was added to platelets 2 minutes prior to challenge with bacteria (100 μg/mL). Other conditions as in Fig 1. Data represent one of three experiments.

The role of platelet fibrinogen in aggregation of human platelets induced by mutant Re595 was substantiated further by using synthetic peptide analogs of fibrinogen sites present on the γ and α chains of human fibrinogen and involved in interaction with platelet receptors.[22] As shown in Fig 4, the peptide analog of the human fibrinogen γ chain (γ 400–411)[25] blocked platelet aggregation induced by mutant Re595 with 50% inhibition (IC_{50}) at 160 μmol/L. The acetylated form of the same peptide, which is not inhibitory toward fibrinogen receptor on human platelets,[25] had no effect on platelet aggregation (results not shown). The synthetic peptide analog of the site on the α chain of human fibrinogen (α 572–575) and of the cell adhesion site of fibronectin[24] also inhibited platelet aggregation with IC_{50} of 60 μmol/L. Neither synthetic peptide inhibited secretion of serotonin induced by mutant Re595. These experiments indicate that platelet aggregation induced by mutant Re595 involves the interaction of platelet fibrinogen with receptors that are blocked by synthetic peptides known to inhibit binding of exogenous plasma fibrinogen.

Role of divalent cations. Addition of 5 mmol/L of EDTA or 5 mmol/L of EGTA to the incubation mixture of platelets and bacteria abolished the aggregation response of platelets, whereas secretion of ^{14}C-serotonin was 39% and 43%, respectively, as compared with a control of 57% (Fig 5). When Mg^{2+} EGTA (5 mmol/L) was used to chelate calcium, while magnesium was maintained at the physiologic level,[39] aggregation was reduced (Tmax 13 *v* 40 in control; slope value 2 *v* 9 in control) and secretion of ^{14}C-serotonin was 33% *v* control 59%.

Because the function of platelet membrane glycoproteins IIb and IIIa depends on calcium, its chelation for 1 hour at 37 °C leads to irreversible modification of the glycoprotein heterodimer.[40,41] After incubation of platelets with 5 mmol/L of EDTA or 5 mmol/L of EGTA under such conditions, the aggregation response was abolished, whereas secretion of ^{14}C-serotonin was only moderately reduced to levels similar to those observed in the previous experiments (37% with

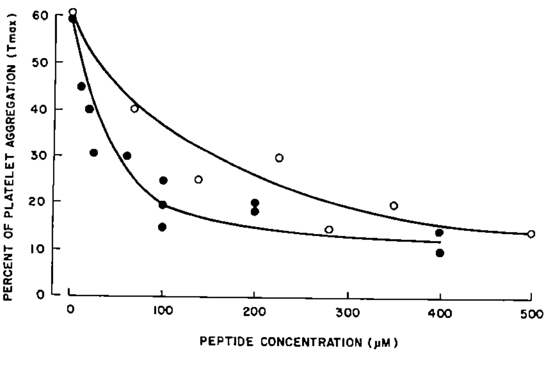

Fig 4. Inhibitory effect of synthetic peptide analogs on aggregation of human platelets induced by mutant Re595 of *Salmonella minnesota*. Synthetic peptide analog of human fibrinogen γ chain cell adhesion site (γ 400–411) (○—○) or tetrapeptide RGDS analogous to cell adhesion site of fibronectin and human fibrinogen α chain (α 572–575) (●—●) were added to human platelets 2 minutes before challenge with bacteria (100 μg/mL). Other conditions as in Fig 1. Cumulative data points from three different experiments.

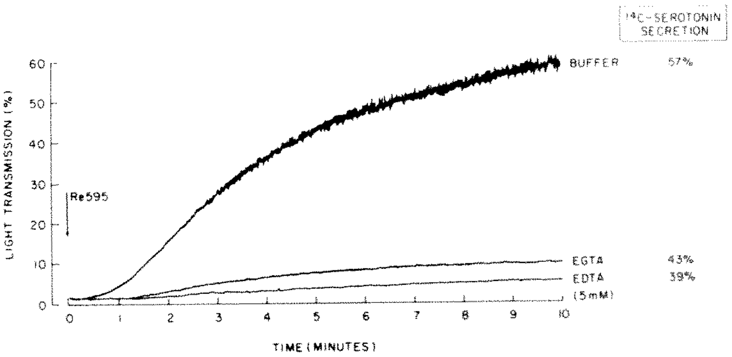

Fig 5. The effect of EDTA and EGTA on platelet aggregation and secretion of ^{14}C-serotonin induced by mutant Re595. EDTA or EGTA (5 mmol/L) was mixed with the platelet suspension 1 minute prior to addition of mutant Re595 (arrow). Other conditions as in Fig 1.

chelators *v* 47% in the control). Preincubation of mutant Re595 with the same chelators did not affect the mutant's ability to induce aggregation of human platelets and secretion of ^{14}C-serotonin. These results indicate that only the aggregation response of human platelets induced with mutant Re595 is prevented by chelation of divalent cations, primarily calcium, and that secretion of serotonin is maintained even under conditions that lead to irreversible modification of the platelet membrane complex of glycoproteins IIb and IIIa.

Phosphorylation of platelet proteins induced by mutant Re595. Stimulation of platelets includes protein kinase C activation manifested by phosphorylation of a 47-kd protein in platelets, myosin light chain kinase activation resulting in phosphorylation of the 20-kd light chain of myosin, and cyclooxygenase activity resulting in transformation of arachidonic acid into thromboxane A_2 through prostaglandin endoperoxides (PGG_2 and PGH_2).[42] These pathways were examined in human platelets after challenge with *S minnesota*. Mutant Re595 induced phosphorylation of a 47-kd protein with relatively more radiolabel incorporation than in a 20-kd protein (Fig 6). The phosphorylation of both 47-kd and 20-kd proteins was rapid, reaching a peak between 60 and 120 seconds, and was followed by dephosphorylation of both proteins. During this period, secretion of ^{14}C-serotonin was continuously increasing even at 15 minutes (Fig 7). Under the same experimental conditions, the smooth strain S218 of *S minnesota* did not induce phosphorylation of the 47-kd and 20-kd proteins above the level observed in control preparation with buffer.

Prostaglandin pathway stimulation by mutant Re595. When the involvement of the prostaglandin pathway was examined, we observed that mutant Re595 induced release of 7.7% ± 1.4 (SEM) of ^{14}C-arachidonic acid from the prelabeled platelet phospholipid pool, whereas the smooth strain S218 caused 1.3% ± 0.4% release and control (buffer) 0.9% ± 0.2%. In subsequent experiments using HPLC for measurement of radiolabeled arachidonic acid metabolites, mutant Re595 induced release of 9% of total ^{3}H-arachidonic acid incorporated into the platelet phospholipid pool. Release of ^{3}H-arachidonic acid was time-dependent and was paralleled by secretion of ^{14}C-serotonin. The arachidonic acid released from the platelet phospholipid pool constituted 53% of the total pool of released radiolabeled products. Among the remaining metabolites of ^{3}H-arachidonic acid, products of cyclooxygenase activity (thromboxane B_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and HHT) constituted 20%, malonyldialdehyde constituted 5%, products of lipooxygenase activity (HETE) constituted 10%, and an unidentified radioactive fraction eluting after the arachidonic acid peak constituted 12%. This pattern of distribution of ^{3}H-arachidonic acid metabolites was similar to that induced by thrombin (0.5 U/mL) except that half as much HETE production was observed in platelets stimulated with mutant Re595.

When acetylsalicylic acid (10^{-4} mol/L) was added to platelets prior to challenge with mutant Re595, generation of cyclooxygenase products and malonyldialdehyde was completely suppressed. This inhibition was paralleled by the inhibition of ^{14}C-serotonin secretion. Another nonsteroidal antiinflammatory agent, indomethacin (5×10^{-4}), also reduced secretion of ^{14}C-serotonin. Hirudin (2 U/mL), while completely blocking the thrombin-induced activation of platelets, remained without effect on changes induced by mutant Re595.

Involvement of core glycolipid in the activation of human platelets by mutant Re595. Because the structure of "incomplete" lipopolysaccharide in mutant Re595 is well established as being composed of Lipid A and KDO,[43] experiments were designed to examine the role of these constituents of glycolipid in its interaction with human platelets. Lipid A is

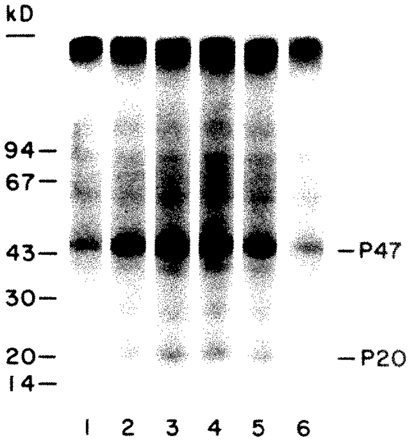

Fig 6. Phosphorylation of human platelet proteins induced by mutant Re595 of *Salmonella minnesota* (125 μg/mL). ^{32}P-Labeled platelets were stirred at 37 °C with buffer, lane 1; or with mutant Re595 for 30 seconds, lane 2; 60 seconds, lane 3; 2 minutes, lane 4; 5 minutes, lane 5; and 15 minutes, lane 6. P47 represents protein of mol wt 47,000; P20 represents myosin light chain.

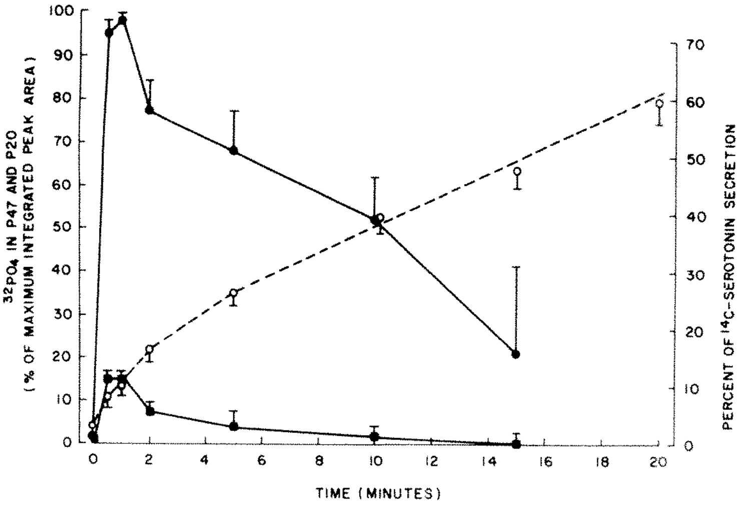

Fig 7. Time course of phosphorylation of platelet protein of mol wt 47,000 (P47, ●—●) and myosin light chain (P20, ■—■) as compared with secretion of ^{14}C-serotonin (O---O) induced by mutant Re595 (125 μg/mL). Percentage of maximum integrated peak area used to express degree of $^{32}PO_4$ incorporation as a measure of phosphorylation refers to the maximal value attained by phosphorylated P47 at 1 minute. Data points were obtained after subtracting the phosphorylation values in the control system containing platelets and buffer. The vertical bars represent ± SEM of four experiments. Secretion of ^{14}C-serotonin was measured in parallel using a platelet preparation not labeled with $^{32}PO_4$. The vertical bars represent ± SEM of eight experiments.

known to form complexes with an extract of amebocytes obtained from the horseshoe crab, *Limulus polyphemus*.[43] As shown in Table 1, mutant Re595 complexed with Limulus extract lost its proaggregatory activity toward human platelets, indicating that cell wall-bound Lipid A in mutant Re595 is involved in its interaction with the platelet membrane. Likewise, secretion of ^{14}C-serotonin was inhibited. Furthermore, polymyxin B, another inhibitor known to form a complex with Lipid A and inhibit its toxic effect,[36] decreased the aggregation of human platelets and secretion of ^{14}C-serotonin by mutant Re595 (Table 1).

Mild acid hydrolysis used to modify the glycolipid markedly reduced the ability of mutant Re595 to induce secretion of serotonin and aggregation (Fig 8A and B). This was accompanied by a decrease in the concentration of KDO associated with bacteria as measured by the thiobarbituric acid assay. A corresponding increase of free KDO in the supernatant during acid hydrolysis of mutant Re595 was observed. Alkaline hydrolysis of ester-linked fatty acids in Lipid A of mutant Re595 also abolished its platelet activating effect (results not shown).

Inhibitory effect of oligosaccharides. Whereas the rough mutant *S minnesota* Re595 induced secretion of ^{14}C-serotonin and aggregation of human platelets, the smooth strain *S minnesota* S218 was inactive (Figs 1 and 2). Because the main difference between these two strains involved the core oligosaccharide and *O*-polysaccharide side chains, this portion of the lipopolysaccharide structure was removed from strain S218 by acid hydrolysis. The obtained oligosaccharide fraction, when added to platelets that were subsequently challenged with mutant Re595, inhibited platelet aggregation. As shown in Fig 9, this effect was concentration-dependent, and 50% inhibition of platelet aggregation was observed at 18 μg/mL of the oligosaccharide fraction. Secretion of ^{14}C-serotonin induced by Re595 was also inhibited by the oligosaccharide fraction but at a higher concentration (50% inhibition was observed at 240 μg/mL). Similarly, the oligosaccharide fraction obtained from isolated lipopolysaccharide *S minnesota* 9700 (Difco) inhibited platelet aggregation and secretion of ^{14}C-serotonin induced by mutant Re595 (results not shown). Thus, oligosaccharides derived from complete endotoxic lipopolysaccharides are capable of interfering with cell wall-bound glycolipid reactivity toward human platelets.

Table 1. Effect of Lipid A Inhibitors on Interaction of Mutant Re595 of *Salmonella Minnesota* With Human Platelets

Inhibitor Added to Mutant Re595	Platelet Aggregation (T_{max}/Slope)	^{14}C-Serotonin Secretion (%)
Buffer (control)	55/33	77
Limulus amebocyte lysate	0/0	12
Polymyxin B	5/1	39

DISCUSSION

Endotoxic lipopolysaccharide is known to have multiple interactions with the complement system,[2] cross-reactive Re antibodies,[44] the kinin system,[45] and high-density lipoproteins.[46] Therefore, our experiments were done in a plasma-free system in order to dissect the direct effects of gram-negative bacteria on the platelet membrane. Mutant Re595 was an effective inducer of platelet secretion and aggregation, which required fibrinogen secreted from platelet α-granules because anti-fibrinogen Fab antibody fragments inhibited platelet aggregation. Fibrinogen secreted from platelet α-granules or present in plasma is bound to specific platelet receptors exposed on the membrane of activated platelets, thereby linking two or more platelets together to form an aggregate.[47] The exposure of receptors for fibrinogen by mutant Re595 required ADP since apyrase prevented platelet aggregation. Blockade of fibrinogen receptors with synthetic peptide analog corresponding to the sequence of γ chain 400–411[25] and with the synthetic peptide analog corresponding to the sequence of α chain 572–575 of human fibrinogen and the cell adhesion site of fibronectin[24] prevented platelet aggregation without affecting secretion of serotonin. This indicates that fibrinogen secreted from platelet α-granules interacts with the platelet membrane receptor for fibrinogen during aggregation induced by mutant Re595.

Our results indicate that activation of human platelets by

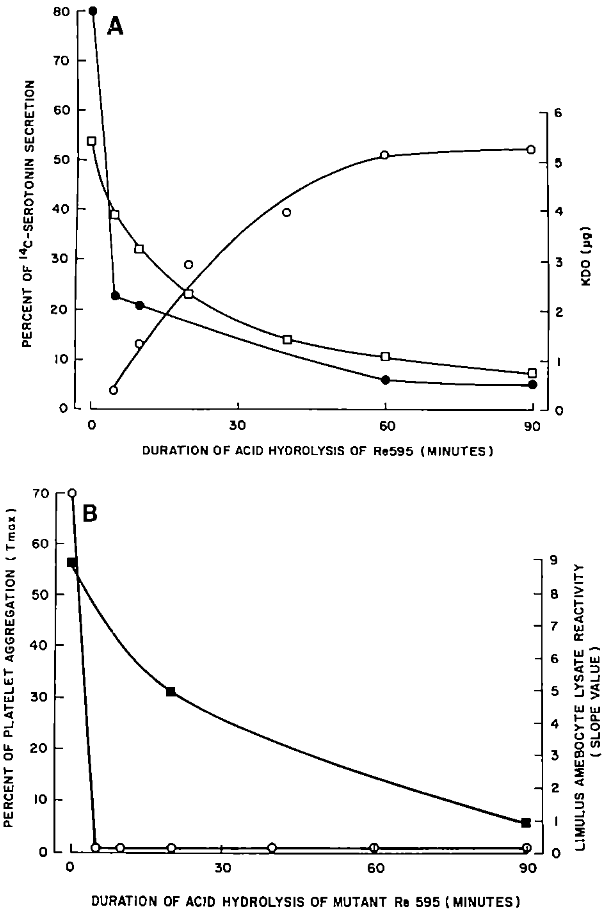

Fig 8. Loss of cell wall-bound 2-keto-3-deoxyoctonate (KDO) from mutant Re595 during acid hydrolysis and decrease in its reactivity toward *Limulus* amebocyte lysate and toward human platelets. Mutant Re595 was subjected to acid hydrolysis for 90 minutes, during which time samples were obtained and centrifuged to determine residual KDO content of bacterial pellet, free KDO content in supernatant and reactivity of bacterial pellet toward *Limulus* amebocyte lysate and human platelets. Panel A: KDO content in mutant Re595 (□——□) and in free supernatant (○——○) and reactivity toward platelets as measured by secretion of ^{14}C-serotonin (●——●). The content of KDO is expressed in microgram per milligram of dry weight of Re595 subjected to acid hydrolysis. Panel B: reactivity of mutant Re595 toward human platelets as measured by their aggregation (○——○) and toward *Limulus* amebocyte lysate (■——■). Data represent one of two experiments.

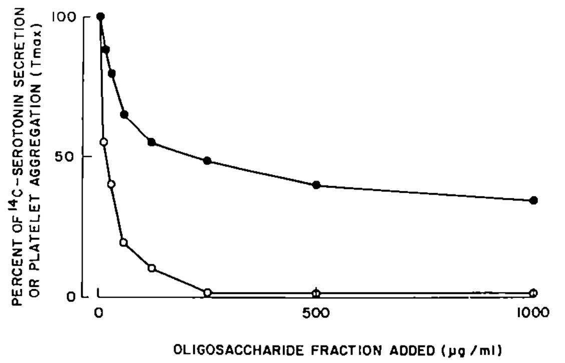

Fig 9. Inhibitory effect of oligosaccharide fraction isolated from smooth strain S218 of *Salmonella minnesota* on platelet aggregation (○——○) and ^{14}C-serotonin secretion (●——●) induced by rough mutant Re595. Oligosaccharide fraction obtained after acid hydrolysis of smooth strain S218 was added to human platelets (2 × 10^8/mL) challenged with rough mutant Re595 (100 µg/mL). Other conditions as in Fig 1. Cumulative data from two experiments.

rough mutant Re595 of *S minnesota* involves stimulatory pathways that are associated with protein kinase C, myosin light chain kinase, and cyclooxygenase, and resembles the pattern of response evoked by collagen.[48] A hitherto unrecognized effect of gram-negative bacteria on human platelets is phosphorylation of the 47-kd protein. This effect suggests that endotoxic glycolipid activates platelet protein kinase C, an enzyme stimulated by phorbol ester, a known activator of platelets.[49–51] Recently, activation of protein kinase C in a murine macrophage-like cell line by Lipid A, or its precursor Lipid X, was reported.[52]

The interaction of mutant Re595 with human platelets results in generation of free arachidonic acid from platelet membrane phospholipids. Conversion of newly generated arachidonic acid into endoperoxides and thromboxane A_2 is essential for the functional effects of mutant Re595 because cyclooxygenase inhibitors (aspirin and indomethacin) block secretion of ^{14}C-serotonin by mutant Re595. Apparently, stimulation of protein kinase C and myosin light chain kinase was not sufficient to cause secretion of ^{14}C-serotonin when the cyclooxygenase pathway was blocked. In such a situation, aspirin does not appear to prevent phosphorylation of the 47-kd protein (J. Grabarek, S. Timmons, and J. Hawiger, unpublished observations). Whether dephosphorylation of 47-kd and 20-kd proteins was due to the activation of a phosphatase(s) remains to be determined. The time course indicates that progressive secretion of ^{14}C-serotonin in this system was accompanied by dephosphorylation of 47-kd protein.

The structural integrity of cell wall-bound glycolipid is required for its activating effect on platelets. Acid hydrolysis results in a loss of reactivity toward platelets. Although KDO is dissociated from Lipid A under such conditions,[32] other changes induced during acid hydrolysis cannot be discounted. Alkaline hydrolysis cleaves ester-linked lauric, palmitic, and myristoxy-myristic acids in Lipid A,[35] resulting in the loss of reactivity of mutant Re595 toward platelets.

Previously, attempts to demonstrate aggregation of human platelets by lipopolysaccharide preparations extracted from various gram-negative bacteria produced divergent results.[9–12,53,54] Our results indicate that association with the cell wall, or part of it, endows the glycolipid with the ability to exert a functional effect on the human platelet membrane. We observed an analogous phenomenon when protein A-bearing staphylococci were shown to interact with human platelets through the IgG Fc receptor to cause their aggregation, whereas isolated protein A (in fluid phase containing IgG) did not.[19] Furthermore, Ginsberg and Henson[55] observed that lipopolysaccharide extracted from mutant Re595 did not induce secretion of serotonin from washed human platelets. It significantly enhanced serotonin secretion when combined with heat-aggregated immunoglobulin G or with immune complexes, however.[55]

The smooth strain S218 of *S minnesota* with a complete core oligosaccharide structure and with *O*-polysaccharide side chains did not induce human platelet aggregation in these studies, although it possesses a cell wall-bound glycolipid. Oligosaccharide fraction isolated from strain S218 and from its lipopolysaccharide was inhibitory toward platelet aggregation induced by mutant Re595. Assuming that the mol wt of the oligosaccharide is ~3,500, the IC_{50} for aggregation and secretion were 5 μmol/L and 69 μmol/L, respectively. This range of concentration is 2 to 3 orders of magnitude lower than the concentration of amino sugars reported to inhibit platelet aggregation induced by other agonists.[56,57] Saba and colleagues showed that complete endotoxic lipopolysaccharides inhibit aggregation of human platelets treated with a number of agonists with the exception of ristocetin-induced agglutination.[58]

It is apparent from the data presented in this and the preceding reports that gram-negative bacteria bear in their endotoxic lipopolysaccharide a structure that is responsible for their interaction with the human platelet membrane to induce secretion and aggregation, as exemplified by mutant Re595. Whether this structure, comprising Lipid A and KDO, is operational in vivo in patients with gram-negative bacteremia awaits direct evidence. Specific antibody against Lipid A can gain access to neutralize its toxic effects and opsonize *Escherichia coli* 0111 during in vivo phagocytosis.[13] Higher titers of cross-reacting Re antibodies against rough mutants of gram-negative bacteria, such as mutant Re595 used in our study, appeared to protect patients from hemodynamic and hematologic derangements complicating gram-negative bacteremia.[44]

In conclusion, a mutant of gram-negative bacteria, possessing cell wall-bound endotoxic glycolipid composed of Lipid A and KDO, is capable of activating human platelets through three mechanisms involving cyclooxygenase, protein kinase C, and myosin light chain kinase. The secretory response of human platelets leads to their aggregation, which can proceed in the absence of plasma fibrinogen. This new attribute of endotoxic glycolipid associated with the cell wall of *S minnesota* Re595 provides a useful model for further study of the structure–function relationship of the proaggregatory effect of endotoxic glycolipid on human platelets and the inhibitory effect of the adjoining oligosaccharides and *O*-polysaccharide side chains.

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REFERENCES

1. Hawiger J· Platelets in Sanarelli-Schwartzman reaction in human sepsis, in deGaetano G, Garattini S (eds): Platelets: A Multidisciplinary Approach New York, Raven Press, 1978, p 321
2. Morrison DC, Ulevitch RJ: The effects of bacterial endotoxins on host mediation systems. A review. Am J Pathol 93:526, 1978
3. Colucci M, Balconi G, Lorenzet R, Pietra A, Locati D, Donati MB, Semeraro N: Cultured human endothelial cells generate tissue factor in response to endotoxin. J Clin Invest 71:1893, 1983
4. Stern D, Bank I, Nawroth P, Cassimeris J, Kisiel W, Fenton JW III, Dinarello C, Chess L, Jaffe E: Self-regulation of procoagulant events on the endothelial cell surface. J Exp Med 162:1233, 1985
5. Wolff SM, Bennet JV: Gram-negative-rod bacteremia (editorial). N Engl J Med 291.733, 1974
6. Cohen P, Gardner FH. Thrombocytopenia as a laboratory sign and complication of gram-negative bacteremic infection. Arch Intern Med 117:113, 1966
7 Hjort PF, Rapaport SI· The Schwartzman reaction: Pathogenetic mechanisms and clinical manifestations. Annu Rev Med 16:135, 1965
8. DesPrez RM, Horowitz HI, Hook EW: Effects of bacterial endotoxin on rabbit platelets I. Platelet aggregation and release of platelet factors *in vitro.* J Exp Med 114:857, 1961
9 Hawiger J, Hawiger A, Steckley S, Timmons S, Cheng C: Membrane changes in human platelets induced by lipopolysaccharide endotoxin. Br J Haematol 35:285, 1977
10. Ausprunk DH, Das J: Endotoxin-induced changes in human platelet membranes: Morphologic evidence. Blood 51:487, 1978
11 Lewis AF, Dickson RC: Platelet aggregation: Induction by means of a complex between endotoxin and copper (Cu^{2+}) Can J Biochem 49:1236, 1971
12. Mueller-Eckhardt CM, Luscher EF: Immune reactions of human blood platelets. IV. Investigations on the problem of an immunologically induced effect of endotoxin on human platelets. Thromb Diath Haemorrh 20.336, 1968
13 Westphal O: Bacterial endotoxins. Int Arch Allergy Appl Immunol 49:1, 1975
14 Osborn MJ, Rick PD, Lehman V, Rupprecht E, Singh M: Structure and biogenesis of the cell wall envelope of gram-negative bacteria. Ann NY Acad Sci 235·52, 1974
15. Elin RJ, Wolff SM Biology of endotoxin. Annu Rev Med 27:127, 1976
16. Wilson GS, Miles A. Topley and Wilson's Principles of Bacteriology, Virology, and Immunity (ed 6). Baltimore, William & Wilkins, 1975, p 934
17 Levin J, Poore TE, Young NS, Margolis S, Zauber NP, Townes SA, Bell WR· Gram-negative sepsis· Detection of endotoxemia with the *Limulus* test. With studies of associated changes in blood coagulation, serum lipids, and complement. Ann Intern Med 76:1, 1972
18. Timmons S, Hawiger J: Separation of human platelets from plasma proteins including factor $VIII_{vWF}$ by a combined albumin gradient-gel filtration method using HEPES buffer. Thromb Res 12:297, 1978
19. Hawiger J, Steckley S, Hammond D, Cheng C, Timmons S, Glick AD, DesPrez RM: Staphylococci-induced human platelet injury mediated by protein A and immunoglobulin G Fc fragment receptor J Clin Invest 63:931, 1979
20. Holmsen H, Setkowsky-Dangelmaier CA· Adenine nucleotide metabolism of blood platelets. X. Formaldehyde stops centrifugation-induced secretion after A23187-stimulation and causes breakdown of metabolic ATP. Biochim Biophys Acta 497:46, 1977
21. Born GVR: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927, 1962
22. Hawiger J, Timmons S, Kloczewiak M, Strong DD, Doolittle RF: Gamma and alpha chains of human fibrinogen possess sites

reactive with human platelet receptor. Proc Natl Acad Sci USA 79:2068, 1982

23. Porter RR: The hydrolysis of rabbit gamma-globulin and antibodies with crystalline papain. Biochem J 73:119, 1959

24. Pierschbacher MD, Ruoshalti E: Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature 309.30, 1984

25. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J· Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the gamma chain. Biochemistry 23:1767, 1984

26. Lyons RJ, Stanford N, Majerus PW: Thrombin-induced protein phosphorylation in human platelets. J Clin Invest 56:924, 1975

27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

28 Gluvna JA: Gel scanning on the Gilford response. Gilford Technical Insights, No 8, 1984

29 Bills TK, Smith JB, Silver MJ: Metabolism of ^{14}C-arachidonic acid by human platelets Biochim Biophys Acta 424:303, 1976

30. Russell FA, Deykin D: The use of high pressure liquid chromatography (HPLC) for the separation of radiolabeled arachidonic acid and its metabolites produced by thrombin-treated platelets I The validation of the technique. Prostaglandins 18:11, 1979

31. Bergmeyer HV, Bernt E: Lactic dehydrogenase, in Bergmeyer HV (ed): Methods of Enzymatic Analysis (ed 2), vol 2. Orlando, Fla, Academic Press, 1974, pp 574–576

32. Osborn MJ: Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhinurium*. Proc Natl Acad Sci USA 50.499, 1963

33. Waravdekar VS, Saslaw LD: A sensitive colorimetric method for the estimation of 2-deoxy sugars with the malonylaldehyde-thiobarbituric acid reactions. J Biol Chem 234:1945, 1959

34. Levin J· The reaction between bacterial endotoxin and amebocyte lysate, in Cohen E (ed): Biomedical Applications of the Horseshoe Crab (*Limulidae*). New York, Liss, 1976, p 131

35. Beckman I, Ludertiz O, Westphal O: Zur immunochemie der somatischen antigene von Enterobacteriaceae. IX. Serologische typisierung von Salmonella-R-Antigenen. Biochem Z 339:401, 1964

36. Morrison DC, Jacobs DM. Binding of polymyxin B to the Lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13:813, 1976

37. Traverso-Cori A, Traverso S, Reyes H: Different molecular forms of potato apyrase. Arch Biochem Biophys 137:133, 1970

38. Tollefsen DM, Majerus PW: Inhibition of human platelet aggregation by monovalent anti-fibrinogen antibody fragments. J Clin Invest 55:1268, 1975

39. DesPrez RM, Bryan CS, Hawiger J, Colley DG: Function of the classical and alternate pathways of human complement in serum treated with ethylene glycol tetraacetic acid and MgCl$_2$-ethylene glycol tetraacetic acid. Infect Immun 11:1235, 1975

40. Zucker MB, Varon D, Masiello NC, Karpatkin S: The combining ability of glycoproteins IIb, IIIa and Ca^{2+} in EDTA-treated nonaggregable platelets. Thromb Haemost 50:848, 1983

41. Shattil SJ, Brass LF, Bennett JS, Pandhi P: Biochemical and functional consequences of dissociation of the platelet membrane glycoprotein IIb–IIIa complex. Blood 66.92, 1985

42. Nishizuka Y: Turnover of inositol phospholipids and signal transduction Science 225:1365, 1984

43. Galanos C, Freudenberg MA, Luderitz O, Rietschel ET, Westphal O. Chemical, physicochemical and biological properties of bacterial lipopolysaccharides, in Cohen E (ed) Biomedical Applications of the Horseshoe Crab (*Limulidae*). New York, Liss, 1976, p 321

44. McCabe WR, Kreger BE, Johns M Type-specific and cross-reactive antibodies in gram-negative bacteremia. N Engl J Med 287:261, 1973

45. Nies A, Melmon KL: Variation in endotoxin-induced kinin production and effect between the rabbit and the rhesus monkey. Am J Physiol 225:230, 1973

46. Ulevitch RJ, Johnston AR, Weinstein DB: New function for high density lipoproteins. Isolation and characterization of a bacterial lipopolysaccharide-high density lipoprotein complex formed in rabbit plasma. J Clin Invest 67:827, 1981

47. Hawiger J, Kloczewiak M, Timmons S: Platelet-receptor mechanisms for adhesive macromolecules, in Oates J, Hawiger J, Ross R (eds). Interaction of Platelets with the Vessel Wall, Clinical Physiology Series, American Physiological Society. Baltimore, Williams & Wilkins, 1985, p 1

48. Hawiger J, Steer M, Salzman E: Intracellular Regulatory Processes in Platelets, in Colman R, Hirsh J, Marder V, Salzman E (eds): Hemostasis and Thrombosis. Basic Principles and Clinical Practice (ed 2). Philadelphia, Lippincott (in press)

49. Zucker MB, Troll W, Belman S: The tumor-promoter phorbol ester (12-*O*-tetradeconyl-phorbol-13-acetate), a potent aggregating agent for blood platelets. J Cell Biol 60.325, 1974

50. White JG, Rao GHR, Estensen RD: Investigation of the release reaction in platelets exposed to phorbol myristate acetate. Am J Pathol 75:301, 1974

51. Nishizuka Y: The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308:693, 1984

52. Wightman PD, Raetz CRH: The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J Biol Chem 259:10048, 1984

53 Ream VI, Deykin D, Gurewich V, Wessler S: The aggregation of human platelets by bacterial endotoxin J Lab Clin Med 66:245, 1965

54. Nagayama M, Zucker MB, Beller FK: Effect of a variety of endotoxins on human and rabbit platelet function. Thromb Diath Haemorrh 26·467, 1971

55 Ginsberg MH, Henson PM: Enhancement of platelet response to immune complexes and IgG aggregates by Lipid A-rich bacterial lipopolysaccharides. J Exp Med 147:207, 1978

56. Houston DS, Gerrard JM, McCrea J, Glover S, Butler A. The influence of amines on various platelet responses. Biochim Biophys Acta 734:267, 1983

57. Kinlough-Rathbone RL, Packham MA, Mustard JF: Effect of amino sugars on platelet aggregation and on fibrinogen binding. Thromb Haemost 52:75, 1984

58. Saba HI, Saba SR, Morelli G, Hartmann RC: Endotoxin-mediated inhibition of human platelet aggregation. Thromb Res 34:19, 1984

Decrease in Subunit Aggregation of Phosphoribosylpyrophosphate Synthetase: A Mechanism for Decreased Nucleotide Concentrations in Pyruvate Kinase–Deficient Human Erythrocytes

By Charles R. Zerez, Neil A. Lachant, and Kouichi R. Tanaka

Pyruvate kinase (PK)-deficient RBCs have several unexplained metabolic abnormalities, such as decreased concentrations of total adenine nucleotides (AMP, ADP, and ATP) and total (oxidized and reduced) nicotinamide adenine dinucleotide (NAD). Because 5-phosphoribosyl-1-pyrophosphate (PRPP) is an intermediate in the synthesis of adenine nucleotides and NAD, we investigated PRPP synthetase (PRPPS), the enzyme responsible for PRPP synthesis. This enzyme is regulated, in part, by changes in its state of subunit aggregation. The proportion of aggregated PRPPS can be altered in vitro by ATP and 2,3-diphosphoglycerate (DPG). Because PK-deficient RBCs have decreased ATP and increased DPG concentrations, we examined the state of subunit aggregation of PRPPS in RBCs from normal and PK-deficient subjects, using gel permeation chromatography. Young normal RBCs have more aggregated PRPPS than do older RBCs. In contrast, due to their decreased ATP and increased DPG concentrations, PK-deficient RBCs contain less aggregated PRPPS than do RBCs of comparable age without PK deficiency. These data suggest that PRPPS should be less active in vivo in PK-deficient RBCs. This may play a key role in mediating the decreases in total adenine nucleotide and total NAD concentrations in these RBCs.

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PYRUVATE KINASE (PK)-deficient RBCs have a decreased concentration of adenosine 5′-triphosphate (ATP), which is caused by their inability to regenerate ATP from ADP due to decreased PK activity.[1] Diminished ATP regeneration in PK-deficient RBCs should cause an accumulation of ADP or AMP. Because most studies have found no consistent increases in ADP and AMP concentration in PK-deficient RBCs,[1] however, there may be secondary metabolic defects in addition to PK deficiency. The lack of increase in ADP and AMP concentration results in a decreased total adenine nucleotide (AMP, ADP, and ATP) concentration. Grimes and co-workers[2] determined the ATP, ADP, and AMP concentrations in RBCs from four splenectomized patients with severe PK deficiency and marked reticulocytosis. Using the data of Grimes and co-workers,[2] we calculated that the mean total adenine nucleotide concentrations in their normal, high reticulocyte, and PK-deficient RBCs are 119, 137, and 84 μmol/100 mL RBCs respectively. Because the decrease in total adenine nucleotide content cannot be attributed to decreased ATP regeneration from ADP, it must be due to changes in the rate of synthesis or degradation of adenine nucleotides.

PK-deficient RBCs are also characterized by a marked decrease in total (oxidized and reduced) nicotinamide adenine dinucleotide (NAD) concentration.[2] We recently reported that this is caused in part by impaired NAD synthesis due to decreased ATP regeneration.[3] Because NAD synthesis requires several metabolites in addition to ATP, however, the unavailabilty of any one of these metabolites may also contribute to decreased total NAD concentration in PK-deficient RBCs.

5-Phosphoribosyl-l-pyrophosphate (PRPP) is an intermediate that is required for the synthesis of adenine nucleotides from adenine via the salvage pathway[4] and for the synthesis of NAD from nicotinic acid.[5] PRPP is synthesized from ATP and ribose-5-phosphate in a reaction catalyzed by PRPP synthetase (PRPPS), an enzyme with complex regulatory properties that include feedback inhibition by several nucleotides that require PRPP for their biosynthesis (eg, NAD and ADP).[6,7] Human RBC PRPPS is a multimeric enzyme that exists in various states of subunit aggregation ranging in mol wt between 65,000 (2 subunits) and 1,040,000 daltons (32 subunits).[8-10] PRPPS subunit aggregation is dependent on several compounds: ATP, ADP, AMP, PRPP, and Mg^{2+} promote association of PRPPS subunits, whereas 2,3-diphosphoglycerate (DPG) promotes their dissociation.[9,10] Because disaggregated forms of PRPPS (1, 2, 4, and 8 subunits) have <4% of the activity of aggregated forms (16 and 32 subunits),[11] in vivo PRPPS activity should be regulated by the association and dissociation of its constituent subunits.

Because PK-deficient RBCs have a decreased concentration of ATP and an increased concentration of DPG, we examined PRPPS in these RBCs to determine whether it plays a role in the decrease in total adenine nucleotide and total NAD concentrations. Our initial studies did not demonstrate a significant difference in PRPPS activity in crude hemolysate from normal, high reticulocyte, and PK-deficient RBCs. Therefore, we examined the state of PRPPS subunit aggregation in hemolysates from PK-deficient RBCs to determine if in vitro modifiers of PRPPS subunit aggregation (ie, ATP and DPG) have the same effects in vivo. The evidence presented in this report suggests that PRPPS subunits are in a relatively less aggregated state in PK-deficient RBCs due to the increased DPG and decreased ATP concentrations in these cells. We propose that the resulting in vivo decrease in PRPPS activity may be a primary cause of decreased total adenine nucleotides and a

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contributing cause of decreased total NAD in PK-deficient RBCs.

MATERIALS AND METHODS

Materials Sephadex G-200 was purchased from Pharmacia Inc, Piscataway, NJ. All other reagents were purchased from Sigma Chemical Co, St Louis

Methods. After informed consent was obtained, blood was obtained by routine venipuncture from persons with PK deficiency and anticoagulated with heparin (15 U/mL of whole blood). Blood from normal subjects and from persons with autoimmune hemolytic anemia (AIHA) or hemolytic anemia of unknown etiology (HA) served as appropriate controls. The separation and washing of erythrocytes have been described previously[12] Hemolysates were prepared by adding 2 vol of distilled water to 1 vol of packed erythrocytes, freezing in an acetone–dry-ice bath, and thawing in a room temperature water bath The hemolysate was centrifuged at 40,000 g for 10 minutes to remove stroma and immediately subjected to gel permeation chromatography on a Sephadex G-200 column. The conditions of chromatography were those of Yip and colleagues,[10] except that the column buffer contained only 2.0 mmol/L of $MgCl_2$. Because the proportion of aggregated PRPPS is known to decrease with storage,[10] all samples were applied to the Sephadex G-200 column within 60 to 90 minutes of phlebotomy.

Two peaks of PRPPS activity were eluted from the Sephadex G-200 column. The first corresponded to aggregated forms of the enzyme and eluted in the void volume, whereas the second corresponded to disaggregated forms of the enzyme and coeluted with hemoglobin.[10] Disaggregated forms of the enzyme could be assayed because they become fully aggregated and, therefore, fully active in the assay mixture due to the presence of saturating concentrations of ATP and Mg^{2+}.[9] Aggregated PRPPS was expressed as the percentage of PRPPS activity in the void volume peak relative to total eluted PRPPS activity.

PRPP synthetase was assayed by measuring the rate of AMP formation using the continuous spectrophotometric method of Valentine and Kurschner.[13] All assay determinations were corrected for ribose-5-phosphate-independent AMP formation. Pyruvate kinase activity was determined as described by Tanaka.[14] Spectrophotometric determinations of ATP[15] and DPG[16] were made using neutralized perchloric acid extracts of whole blood.[17] Hemoglobin concentration was determined using the cyanmethemoglobin method.[17]

Data are expressed as the mean ±1 SD. Student's *t*-tests were performed by standard methods. Correlation coefficients (*r*) were determined using the least-squares method Analysis of covariance was performed by adjusting the percentage of aggregated PRPPS by the intraerythrocytic concentration of ATP and DPG using the following formula: adjusted percentage of aggregated PRPPS = $PAP_n + b\ (I - I_n)$ where PAP_n is the percentage of aggregated PRPPS in the nth sample, b is the slope of the least-squares line of percentage of aggregation *v* ATP or DPG, I is the mean concentration of ATP or DPG in the cohort, and I_n is the concentration of ATP or DPG in the nth sample.[18]

RESULTS

Initial studies dealt with the measurement of PRPPS activity. When corrected for cell size, the mean PRPPS activity of normal (n = 14), high reticulocyte (3.4% to 28%, n = 9), and PK-deficient (n = 4) RBCs were not statistically different at 46.1 ± 7.1, 51.9 ± 14.6, and 45.3 ± 7.6 μmol AMP/h·mL of RBCs, respectively. Our inability to demonstrate a decrease in PRPPS activity in PK-deficient RBCs using the standard assay system is a result of the presence of sufficient ATP and Mg^{2+} concentrations to cause full PRPPS subunit aggregation and full activity.[9] The lack of a significant difference in PRPPS activity between our normal and high reticulocyte groups was in contrast to the results of Valentine and Kurschner, who have shown that PRPPS activity increases slightly with the proportion of reticulocytes.[13] This discrepancy may be due to differences in expressing PRPPS activity, since Valentine and Kurschner expressed their results based on activity per number of RBCs.[13]

Given these results, we subsequently measured the state of PRPPS subunit aggregation. Erythrocytes from 12 normal subjects were found to have 37.7% ± 3.5% of their PRPPS in aggregated forms (Table 1). In contrast, the young RBC population from persons with AIHA or HA had a higher

Table 1. State of Aggregation of RBC PRPPS From Normal Subjects, Individuals With AIHA, HA, and PK Deficiency

Subject	Splenectomy	Reticulocytes (%)	ATP (μmol/g Hb)	DPG (μmol/g Hb)	Aggregated PRPPS (%)	ATP-Adjusted* Aggregated PRPPS (%)	DPG-Adjusted* Aggregated PRPPS (%)
Normal (n = 12)	No	1.1 ± 0.5	3.58 ± 0.45	14.1 ± 1.9	37.7 ± 3.5	37.7 ± 2.7	37.7 ± 3.4
Range		(0.4–2.0)	(3.08–4.44)	(11.5–16.7)	(32.0–43.4)	(32.1–40.8)	(32.6–42.7)
AIHA							
Patient 1	Yes	18.0	2.09	15.0	51.6	69.7	56.3
Patient 2	Yes	9.0	2.65	15.8	53.8	64.3	64.0
Patient 3	Yes	19.0	3.35	15.3	64.0	65.0	70.8
Patient 4	No	28.0	—	—	77.7	—	—
HA							
Patient 1	No	15.0	4.44	11.9	74.0	60.1	57.7
Patient 2	No	10.0	4.58	13.5	90.0	74.2	84.6
PK Deficiency							
Patient 1	Yes	41.0	1.90	25.9	33.4	44.1	39.2
Patient 2	No	7.5	2.54	24.1	43.6	47.5	44.4
Patient 3	No	7.5	2.83	25.8	51.9	52.6	57.4
Patient 4	No	11.0	4.32	19.2	60.5	45.3	47.8

PRPPS, 5-phosphoribosyl-1-pyrophosphate synthetase, AIHA, autoimmune hemolytic anemia; HA, hemolytic anemia of unknown etiology; PK, pyruvate kinase; DPG, 2,3-diphosphoglycerate.

*Adjustments were performed using analysis of covariance.

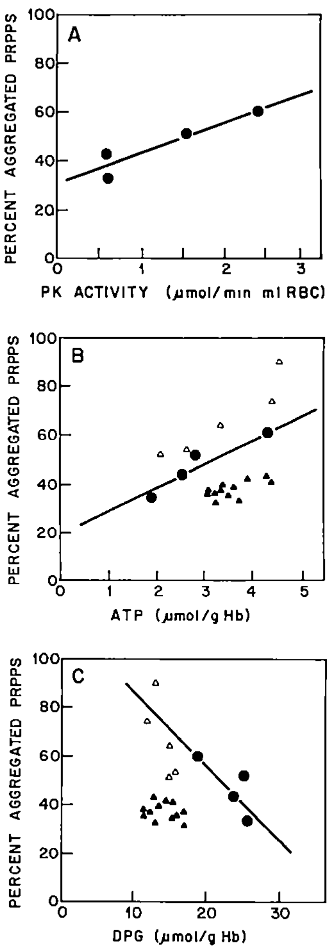

Fig 1. The relationship between the proportion of aggregated phosphoribosylpyrophosphate synthetase (PRPPS) and (A) pyruvate kinase (PK) activity, (B) ATP concentration, and (C) 2,3-diphosphoglycerate (DPG) concentration in RBCs from normal (▲), high reticulocyte (△) and pyruvate kinase-deficient (●) individuals.

proportion of PRPPS in aggregated forms (Table 1). The mean intraerythrocytic concentrations of ATP and DPG in RBCs from the high reticulocyte cohort (n = 5) were 3.42 ± 1.09 and 14.3 ± 1.6 μmol/g hemoglobin, respectively. These values were not significantly different from the mean ATP and DPG concentrations in RBCs from the normal cohort (Table 1).

The proportion of aggregated PRPPS increased in a nonlinear manner with the number of reticulocytes in the RBC sample (Table 1). PRPPS aggregation in the high reticulocyte cohort appeared to be highly dependent on the intraerythrocytic ATP concentration (Table 1, Fig 1B). RBCs from PK-deficient patients 2, 3, and 4 who had not been splenectomized (7.5% to 11% reticulocytes) also had more of their PRPPS in aggregated forms as compared with RBCs from normal subjects ($P < .02$) (Tables 1 and 2). RBCs from the PK-deficient patients had less aggregated PRPPS than did RBCs from AIHA or HA patients with a comparable degree of reticulocytosis, however ($P < .09$) (Tables 1 and 2). What is more significant is that RBCs from PK-deficient patient 1 with the severest enzyme deficiency, who had undergone splenectomy, had a decreased proportion of aggregated PRPPS (33.4%) as compared with either the high reticulocyte RBCs or the RBCs of most normal subjects (Table 1). Presumably, in the absence of the spleen in PK-deficient patient 1, a greater number of very defective RBCs were in circulation.

The proportion of aggregated PRPPS in PK-deficient RBCs correlated positively with PK activity ($r = .90$) (Fig 1A) and intraerythrocytic ATP concentration ($r = .95$) (Fig 1B) and negatively with intraerythrocytic DPG concentration ($r = -.75$) (Fig 1C). The percentage of aggregated PRPPS in normal and high reticulocyte RBCs also correlated positively with intraerythrocytic ATP concentration ($r = .64$ and .94, respectively) (Fig 1B). Furthermore, in high reticulocyte RBCs, there was a negative correlation between the proportion of aggregated PRPPS and intraerythrocytic DPG concentration ($r = -.68$), whereas in normal RBCs little correlation existed between the proportion of aggregated PRPPS and intraerythrocytic DPG concentration ($r = -.23$) (Fig 1C). Because the proportion of aggregated PRPPS appeared to be dependent on the percentage of reticulocytes, the intraerythrocytic ATP concentration, and the intraerythrocytic DPG concentration, an analysis of covariance was performed to adjust the proportion of aggregated PRPPS for differences in ATP and DPG concentrations in normal, high reticulocyte, and PK-deficient RBCs (Table 1). Although there was no statistically significant difference in unadjusted PRPPS aggregation between the high reticulocyte and PK-deficient cohorts ($P < 09$), there were statistically significant differences in both the ATP-adjusted percentage of PRPPS aggregation ($P < .001$) and the DPG-adjusted percentage of PRPPS aggregation ($P < .025$) between these cohorts (Table 2). Thus, when adjusted

Table 2. Statistical Analysis of PRPPS Aggregation in RBCs From Normal Subjects, Individuals With Reticulocytosis, and PK Deficiency

	Aggregated PRPPS (%)		
	Unadjusted	ATP-Adjusted*	DPG-Adjusted*
Mean ± SD			
Group I, normal (n = 12)	37.7 ± 3.5	37.7 ± 2.7	37.7 ± 3 4
Group II, high reticulocyte (n = 5)	66.7 ± 15.8	66.7 ± 5.4	66.7 ± 11 6
Group III, PK deficient (n = 4)	47.4 ± 11.6	47 4 ± 3.8	47.4 ± 7.7
t Test analysis			
I *v* II	$P < .001$	$P < .001$	$P < .001$
II *v* III	$P < .09$	$P < .001$	$P < .025$
III *v* I	$P < 02$	$P < .001$	$P < 005$

*Adjustments were performed using analysis of covariance. Abbreviations as in Table 1.

for either the ATP or DPG concentration, the proportion of aggregated PRPPS in PK-deficient RBCs was significantly lower than the proportion of aggregated PRPPS in high reticulocyte RBCs (Table 2).

Given these relationships, we attempted to mimic PK deficiency in intact normal RBCs in vitro to determine the effect this has on PRPPS subunit aggregation. Freshly obtained normal RBCs were ATP-depleted by incubation with 100 mmol/L of 2-deoxyglucose for 1 hour, conditions that do not cause a significant depletion of DPG.[19] The RBC ATP content decreased by 75% after this treatment. Instead of demonstrating a shift of PRPPS to the dissociated state, however, almost no PRPPS activity was recovered in the eluate (data not shown). We subsequently determined the in vitro effects of ATP and DPG on the state of PRPPS subunit aggregation in hemolysate from normal RBCs. Using RBCs from a normal subject, the proportion of aggregated PRPPS in untreated hemolysate was 41.8% (Fig 2A). When the same hemolysate was incubated with 1.0 mmol/L of ATP for 1 hour at 37 °C prior to column chromatography, aggregated PRPPS increased to 51.5% (Fig 2B). In contrast, incubation of the same hemolysate with 5.0 mmol/L of DPG for 1 hour at 37 °C prior to column chromatography resulted in a decrease of aggregated PRPPS to 17.8% (Fig 2C). In the latter experiment, fewer total units of PRPPS were eluted from the column because 43% of the original PRPPS activity was lost during the incubation with DPG. To determine whether our system is comparable with that of Yip and colleagues[10] despite our slightly modified experimental conditions, we chromatographed hemolysate using column buffer containing 1.0 mmol/L of ATP. One hundred percent of the PRPPS was found in aggregated forms under these conditions, indicating that our results were comparable with those of Yip and colleagues.[10]

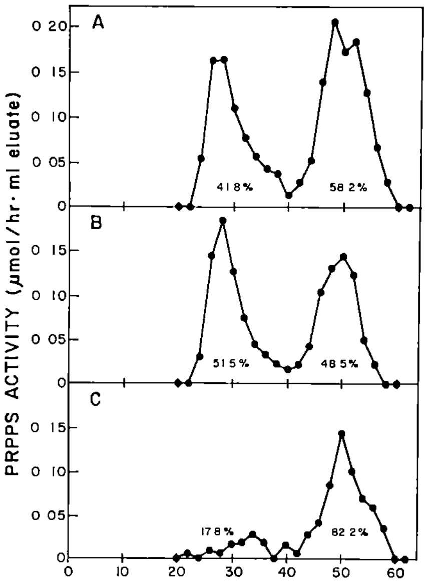

Fig 2. Changes in subunit aggregation of phosphoribosylpyrophosphate synthetase (PRPPS) fron normal RBCs. The first PRPPS activity peak corresponds to aggregated forms of the enzyme while the second peak corresponds to disaggregated forms. A: untreated hemolysate; B: hemolysate incubated with 1.0 mmol/L of ATP for 1 hour at 37 °C; C: hemolysate incubated with 5.0 mmol/L of 2,3-diphosphoglycerate for 1 hour at 37 °C. The percentage of PRPPS activity in each peak relative to total eluted PRPPS activity is shown.

DISCUSSION

The state of subunit aggregation of RBC PRPPS is dependent on two different phenomena. First, it has been shown that ATP induces the subunits of purified PRPPS to aggregate whereas DPG induces aggregated enzyme to dissociate.[9,10] We extended these early findings by demonstrating changes in PRPPS subunit aggregation after relatively short preincubations of crude hemolysate with ATP or DPG. Thus, there was an increase in the proportion of aggregated PRPPS after preincubation of normal hemolysate with ATP and a decrease in the proportion of aggregated PRPPS after preincubation of hemolysate with DPG (Fig 2). The demonstration that 100% of the enzyme was aggregated when ATP was included in the chromatography buffer suggests that our gel permeation system worked in a manner identical to that of Yip and colleagues.[10] Our inability to demonstrate PRPPS disaggregation after ATP depletion with 2-deoxyglucose was due to the loss of PRPPS activity. Such activity losses occur if hemolysate is stored for several days at 4 °C.[10] Because we used only fresh hemolysate in our studies, however, it is possible that incubation of intact RBCs with 2-deoxyglucose may have resulted in loss of PRPPS activity by a mechanism that is not related to ATP depletion. A similar, albeit smaller, loss in activity was observed after hemolysate was preincubated with DPG.

Second, we demonstrated for the first time that the younger RBCs from the high reticulocyte cohort have more aggregated PRPPS than do mature normal RBCs (Table 1). Because the mean intraerythrocytic concentrations of ATP and DPG in the high reticulocyte cohort were not significantly different from normal, the increase in PRPPS subunit aggregation in high reticulocyte RBCs is not due to alterations in the mean concentrations of ATP and DPG by themselves. The proportion of aggregated PRPPS in an individual high reticulocyte RBC sample is highly dependent on intraerythrocytic ATP concentration, however, and somewhat dependent on intraerythrocytic DPG concentration. Thus, these data suggest that there is relatively more aggregated PRPPS in young RBCs de novo and that the proportion of aggregated PRPPS decreases as RBCs mature. It is not known whether erythroblasts have fully aggregated PRPPS. It is known, however, that PRPPS from leukocytes and other tissues examined thus far is fully aggregated and that it does not dissociate into smaller mol wt forms.[10]

The decrease in aggregated PRPPS in PK-deficient RBCs as compared with high reticulocyte RBCs showed a trend toward statistical significance ($P < .09$) (Table 2). Because the proportion of aggregated PRPPS is dependent on cell age and intraerythrocytic ATP and DPG concentrations, changes in any of these parameters will affect the percentage of aggregation. This warranted an analysis of covariance. Thus, when the percentage of aggregation was adjusted for each of the above covariables, the difference in percentage of aggregation between high reticulocyte and PK-deficient RBCs became statistically significant (Table 2). Therefore, it is suggested that PK-deficient RBCs contain less aggregated PRPPS than do RBCs of comparable cell age without PK deficiency. Furthermore, this decrease is most likely caused by the decreased ATP and increased DPG concentrations in PK-deficient RBCs. Because there is less aggregated PRPPS in PK-deficient RBCs than in the high reticulocyte RBCs, our data suggest that PRPPS may be less active in vivo in PK-deficient RBCs than in RBCs of comparable age without enzymopathy. Although the state of PRPPS subunit aggregation in RBCs from PK-deficient patients 2, 3, and 4 was actually higher than that of normal RBCs because of the increased proportion of reticulocytes, the state of PRPPS subunit aggregation in RBCs from PK-deficient patient 1 was at the lower end of the normal range despite the very high proportion of reticulocytes (Table 1). Only PK-deficient patient 1 was comparable with the patients that Grimes and colleagues[2] used for their determination of total adenine nucleotides. Hence, there is good agreement between aggregated PRPPS and total adenine nucleotides in normal, high reticulocyte, and severely PK-deficient RBCs (patient 1 in this study).

The strong correlation between percentage of aggregated PRPPS and intraerythrocytic ATP concentration is due to the marked in vitro aggregating effect of ATP. In contrast, the correlation between PRPPS aggregation and intraerythrocytic DPG concentration is not as strong. This is because a fivefold higher concentration of DPG than ATP is required to affect PRPPS aggregation in vitro.[9] In addition, in intact normal RBCs, DPG is present at a concentration approximately equimolar to that of hemoglobin.[20] Because DPG binds to hemoglobin with high affinity,[20] most of the DPG in normal RBCs would be bound to hemoglobin, and relatively little free DPG would be available to affect PRPPS aggregation. This may account for the low correlation between PRPPS aggregation and intraerythrocytic DPG concentration in normal RBCs (Fig 1C). When the DPG concentration increases to a level that is significantly higher than the hemoglobin concentration, the concentration of free DPG will increase and affect PRPPS aggregation. Thus, only DPG concentrations significantly higher than those found in normal RBCs (14 μmol/g Hb) will contribute to increasing the concentration of free DPG. In our study, this condition is only present in RBCs from our PK-deficient cohort, which may account for the relatively strong correlation between PRPPS subunit aggregation and intraerythrocytic DPG concentration in these RBCs.

Given (a) the strong in vivo correlations between PK activity, intraerythrocytic ATP and DPG concentrations, and the proportion of aggregated PRPPS in PK-deficient RBCs, and (b) the in vitro effect of ATP and DPG on PRPPS subunit aggregation, our data suggest that the decrease in aggregated PRPPS in PK-deficient RBCs is caused by the decreased ATP and increased DPG levels in these RBCs. Lower in vivo PRPPS activity in PK-deficient RBCs would be expected to impair PRPP formation, which would diminish the capacity of these RBCs to synthesize adenine nucleotides and NAD. Therefore, we propose that lower in vivo PRPPS activity in PK-deficient RBCs may be (a) a major cause of decreased total adenine nucleotide concentration, and (b) another cause of decreased total NAD content in addition to diminished ATP regeneration.

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REFERENCES

1. Tanaka KR, Paglia DE: Pyruvate kinase deficiency. Semin Hematol 8 367, 1971

2 Grimes AJ, Meisler A, Dacie JV. Hereditary non-spherocytic haemolytic anaemia. A study of red-cell carbohydrate metabolism in twelve cases of pyruvate-kinase deficiency. Br J Haematol 10.403, 1964

3. Zerez CR, Tanaka KR: Impaired NAD synthesis in pyruvate kinase deficient human erythrocytes. Clin Res 34:98A, 1986 (abstr)

4. Wyngaarden JB, Kelley WN: Gout, in Stanbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown MS (eds): The Metabolic Basis of Inherited Disease. New York, McGraw-Hill, 1983, p 1043

5. Preiss J, Handler P: Biosynthesis of diphosphopyridine nucleotide. II. Enzymatic aspects. J Biol Chem 233:493, 1958

6. Hershko A, Razin A, Mager J Regulation of the synthesis of 5-phosphoribosyl-l-pyrophosphate in intact red blood cells and cell-free preparations. Biochim Biophys Acta 184:64, 1969

7. Fox IH, Kelley WN. Human phosphoribosylpyrophosphate synthetase. Kinetic mechanism and end product inhibition. J Biol Chem 247.2126, 1972

8. Fox IH, Kelley WN· Human phosphoribosylpyrophosphate synthetase. Distribution, purification and properties J Biol Chem 246 5739, 1971

9. Becker MA, Meyer LJ, Huisman WH, Lazar C, Adams WB: Human erythrocyte phosphoribosylpyrophosphate synthetase. Subunit analysis and states of subunit association. J Biol Chem 252:3911, 1977

10. Yip LC, Roome S, Balis ME· In vitro and in vivo age-related modification of human erythrocyte phosphoribosyl pyrophosphate synthetase. Biochemistry 17:3286, 1978

11. Meyer LJ, Becker MA Human erythrocyte phosphoribosylpyrophosphate synthetase. Dependence of activity on state of subunit association. J Biol Chem 252:3919, 1977

12 Zerez CR, Tanaka KR: A continuous spectrophotometric assay for pyrimidine 5'-nucleotidase Anal Biochem 151:282, 1985

13. Valentine WN, Kurschner KK. Studies on human erythrocyte nucleotide metabolism. I. Nonisotopic methodologies. Blood 39:666, 1972

14. Tanaka KR: Pyruvate kinase, in Yunis JJ (ed): Biochemical Methods in Red Cell Genetics New York, Academic Press, 1969, p 167

15. Jaworek D, Gruber W, Bergmeyer HU: Adenosine-5'-triphosphate Determination with 3-phosphoglycerate kinase, in

Bergmeyer HU (ed). Methods of Enzymatic Analysis, vol 4. New York, Academic Press, 1974, p 2097

16. Keitt AS: Reduced nicotinamide adenine dinucleotide-linked analysis of 2,3-diphosphoglyceric acid: Spectrophotometric and fluorometric procedures. J Lab Clin Med 77:470, 1971

17. Beutler E: Red Cell Metabolism. A Manual of Biochemical Methods (ed 3). Orlando, Fla, Grune & Stratton, 1984, p 8

18 Dixon WJ, Massey FJ Jr: Introduction to Statistical Analysis (ed 4). New York, McGraw-Hill, 1983, p 241

19. Kondo T, Beutler E: Depletion of red cell ATP by incubation with 2-deoxyglucose. J Lab Clin Med 94:617, 1979

20. Benesch R, Benesch RE· Intracellular organic phosphates as regulators of oxygen release by haemoglobin. Nature 221:618, 1969

The L4F3 Antigen Is Expressed by Unipotent and Multipotent Colony-Forming Cells but Not by Their Precursors

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Antibody L4F3 is a murine monoclonal antibody that recognizes an antigen expressed on in vitro colony-forming cells, including virtually all CFU-GM, CFU-Meg, BFU-E, and CFU-Mix. In the present study we examined whether cells that do not express the L4F3 antigen include precursors of hematopoietic colony-forming cells. Colony-forming cells were depleted from marrow by treatment with L4F3 and complement. The remaining cells generated CFU-GM, BFU-E, and CFU-Mix when cultured in the presence of irradiated adherent cell layers from long-term marrow cultures. Marrow cells not expressing the L4F3 antigen, which were separated by cell-sorting techniques, were depleted of colony-forming cells but nevertheless generated CFU-GM when cultured over irradiated adherent cell layers. These data suggest that there are marrow precursors that do not express the L4F3 antigen and that give rise to colony-forming cells of multiple types. Negative selection techniques should allow the enrichment of these precursors of colony-forming cells, thereby enabling direct studies of these immature stem cells.

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SURFACE ANTIGENS expressed by cells of the different hematopoietic lineages change as these cells mature.[1,2] Certain of these differentiation antigens are selectively expressed by cells with different lineage commitments and with greater or lesser proliferative potentials. The maturation-linked expression of antigens by granulocyte/monocyte,[3–18] erythroid,[3,19,20] and megakaryocyte/platelet[3,21–23] elements and their committed precursors[3,24–27] have been identified by numerous monoclonal antibodies. Less is known, however, about antigens expressed by the putatively less mature stem cells that are precursors of in vitro colony-forming progenitors since only indirect assays are possible.

The present study examines the expression of the L4F3 antigen by hematopoietic progenitor cells in vitro including the precursors of colony-forming cells We have previously shown that the L4F3 antibody reacts with nearly all granulocyte/monocyte colony-forming cells (CFU-GM) and a portion of erythroid burst-forming cells (BFU-E).[3] In this study, using complement (C′)-mediated cytolysis and separation of cells stained in indirect immunofluorescence assays by fluorescence-activated cell sorting, we show that the L4F3 antibody also reacts with virtually all megakaryocyte (CFU-Meg) and multipotential (CFU-Mix) colony-forming cells. In contrast, we found that the L4F3 antibody does not react with cells that are precursors for CFU-GM, BFU-E, and CFU-Mix detectable in long-term marrow cultures.

MATERIALS AND METHODS

Cells

Under an Institutional Review Board–approved protocol, samples of bone marrow obtained from normal healthy adult donors at the Fred Hutchinson Cancer Research Center were collected in heparinized tissue culture medium Specimens were diluted with an equal volume of RPMI 1640, and bone marrow nucleated cells were separated either as buffy coat cells or by Ficoll-Hypaque (sp gr, 1 077) density gradient centrifugation as previously described [3]

Monoclonal Antibodies

The generation and characterization of the murine monoclonal antibody L4F3 (IgM) has been previously described in detail.[3] The L4F3 antibody used in the cytotoxicity assays was in the form of unfractionated ascites fluid with a cytotoxic titer of antibody $>10^{-3}$ As a control, ascites fluid containing T11D7, a murine monoclonal IgM of irrelevant specificity (antimouse Thy 1.1), was used (hybridoma clone kindly provided by Dr E. Clark, University of Washington, Seattle). For use in immunofluorescence assays the antibodies were purified and conjugated with fluorescein-isothiocyanate (FITC) Monoclonal antibodies L4F3 and T11D7 were partially purified from ascites fluids by precipitation of IgM with 2% (wt/vol) boric acid.[28] To each 1 mL of ascites fluid 20 mL of 2% boric acid was added dropwise with continued stirring on ice. Stirring was continued for 60 minutes, at which time the solution was centrifuged for 20 minutes, 4 °C, 5,000 × g The precipitate was dissolved in phosphate-buffered saline (PBS), pH 7 2 When assayed by microzonal electrophoresis, a single band was seen. Partially purified antibodies were then conjugated with FITC (Research Organics, Cleveland) as previously described.[29] The fluorescein/protein ratio of L4F3-FITC was 6.9:1, and that of T11D7-FITC was 7.9.1 as determined by the OD280/OD495 technique.[29]

Tissue Culture

Long-term marrow cultures. Marrow cells were cultured in either 25-cm² tissue culture flasks (Falcon Labware, Oxnard, Calif) at 10^7 per flask or in Ambitube (Miles Scientific, Naperville, Ill) culture tubes at 0.45 to 10 × 10^6 per tube. As previously described,[30] culture medium consisted of McCoy's 5A medium supplemented with essential and nonessential amino acids and vitamins (GIBCO, Grand Island, NY), 12.5% fetal bovine serum (FBS) (Reheis, Kaukakee, Ill), 12.5% horse serum (GIBCO), 10^{-6} mol/L hydrocortisone (Sigma Chemical Co, St Louis), and 1 mmol/L sodium pyruvate (GIBCO). Cultures were incubated at 37 °C and 5% CO_2

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in a humidified incubator for five days and then transferred to a 33 °C, 5% CO_2-humidified incubator. At weekly intervals cultures were fed. After pipetting the supernatant several times, half of the supernatant fluid was removed along with nonadherent cells and an equal volume of fresh medium added. Cells in culture supernatants were assayed for CFU-GM, BFU-E, or CFU-Mix

Marrow adherent cell layers. Marrow buffy coat cells were cultured at 2×10^7 per 10 mL in 25-cm^2 tissue culture flasks or 3 to 5×10^6 per 2 mL in Ambitube culture tubes as was described. At 3 to 4 weeks after initiation of cultures when a confluent adherent cell layer was present, cultures were irradiated with 1,148 ± 209 cGy from a cesium source (dose rate, 459 ± 83 cGy/min). This treatment resulted in cultures that produced no colony-forming cells even when fresh marrow irradiated with the same dose was added (data not shown).

Colony assays Culture systems for BFU-E and CFU-GM,[3,31] CFU-Meg,[32] and CFU-Mix[31] have previously been described. Cells were cultured at 0.1 to 1.0×10^5 mL, and cultures were counted on day 13 to 15 using an inverted microscope. CFU-GM were identified as colonies with 40 or more cells containing granulocytes, monocytes, or both granulocytes and monocytes. Erythroid bursts were defined as clusters of two or more hemoglobinized colonies after 14 days of culture. These clusters represent progeny of an immature progenitor type.[33] CFU-Mix were colonies containing erythroid and nonerythroid cell types in which there was a single center to the colony. Colonies with two apparent centers, one for erythroid and one for nonerythroid cells, were considered separate colonies and not CFU-Mix. At the cell densities used in these cultures, the majority of CFU-Mix can be expected to be clonal based on studies of G6PD clonality reported by Powell et al.[31]

Cytotoxicity Assay

Cytotoxicity assays were performed as previously described.[3] Briefly, bone marrow cells were suspended at a concentration of 10^7 per mL in RPMI supplemented with 20% (vol/vol) FBS (Hyclone, Logan, Utah) (RPMI–20% FBS) containing a 1.10^3 dilution of antibody-containing ascites fluid. Cells were incubated for 30 minutes at room temperature (RT), at which time an equal volume of a 1:1 dilution of prescreened baby rabbit serum (a source of complement) in RPMI–20% FBS with 10^2 Kunitz U/mL of DNase (GIBCO) was added The cells were resuspended and incubation continued for 60 minutes at RT Cells were washed three times with RPMI and then suspended in RPM1–20% FBS. As a control in these studies, cells were also treated with T11D7, an isotype-identical monoclonal antibody of irrelevant specificity.

Immunofluorescence Assays and Cell Separation by Fluorescence-Activated Cell Sorting

Marrow cells were stained using a 1:20 dilution of directly conjugated L4F3-FITC or T11D7-FITC and then secondarily stained with a 1.20 dilution of an FITC-conjugated goat antiserum to mouse IgM and IgG (Tago, Burlingame, Calif) to enhance fluorescence All antibodyies were centrifuged at 100,000 g at 25 °C for ten minutes in a Beckman airfuge (Beckman Instruments, San Francisco) immediately prior to use to remove antibody complexes.

Cells stained with either L4F3-FITC or irrelevant control T11D7-FITC were analyzed by flow microfluorometry using a FACS-440 (Becton-Dickinson, Oxnard, Calif) L4F3-stained cells with a fluorescence intensity greater that that of 95% of T11D7-stained control cells were selected as positively stained. Positively and negatively staining cells were separated by fluorescence-activated cell sorting (FACS) at a rate of 2 to 4×10^3 cells/s. Cells were collected into tubes containing RPMI–20% FBS. Collected cells were cultured in colony assays as well as in the long-term cultures over irradiated marrow adherent cell layers.

RESULTS

Inhibition of Colony Formation by Treatment With Antibody L4F3 Plus C′

We used C′-dependent cytolysis to examine the reactivity of monoclonal antibody L4F3 with unipotent and multipotent in vitro colony-forming cells. Treatment with L4F3 plus C′ lysed 55% to 75% of marrow nucleated cells when compared with treatment with the control antibody T11D7 as determined by trypan blue dye exclusion in four experiments. Treatment with L4F3 inhibited growth of virtually all immature colony-forming cells including CFU-GM (≥92%), BFU-E (≥95%, bursts with two or more colonies), and CFU-Mix (≥99%) when compared with cells treated with T11D7 (Table 1). In three additional experiments we found that L4F3 treatment also inhibited the growth of CFU-Meg (96% inhibition) (mean ± SEM of 15 replicates per data point. L4F3 + C′ = 0.4 ± $0.3/10^5$; T11D7 + C′ = 9.8 ± $1.9/10^5$). Thus, these results confirm the previously described lysis of CFU-GM and BFU-E by antibody L4F3 and C′[3] and further demonstrate the effects of both CFU-Meg and CFU-Mix.

Separation of Colony-Forming Cells by FACS

The effects on colony-forming cells in cytolytic assays also may be due to the depletion of accessory cells. Therefore, we used FACS to isolate marrow cells expressing the L4F3 antigen. Since the fluorescence intensity of marrow cells stained using L4F3 in indirect immunofluorescent assays is relatively low,the distinction between cells with low levels of antigen expression and unstained cells is difficult. We therefore enhanced the fluorescent signal using L4F3 that was directly conjugated with FITC followed by FITC-conjugated goat antimouse Ig (GAM) antiserum to stain marrow cells. As a control, cells were stained with FITC-conjugated T11D7 followed by the FITC-conjugated GAM Ig antisera. In these experiments, cells were considered positively stained if they had a fluorescence intensity greater than that of 95% of the T11D7-FITC–stained control cells. Cells were separated into L4F3-positive and -negative populations. In a representative experiment shown in Table 2, the L4F3-stained population contained 76% of all sorted marrow cells. The separated positively and negatively reacting cells as well as unseparated cells were cultured for CFU-GM, BFU-E, CFU-Mix, and CFU-Meg.

The L4F3-positive cells contained virtually all of the CFU-Mix, CFU-Meg, and BFU-E, and the majority of CFU-GM were present in the sorted populations. The separation of the majority of BFU-E including multiple-colony clusters (two or more colonies per burst) was also observed in two additional experiments in which 60% and 80% respectively of sorted BFU-E were in the positive populations (data not shown). These results directly demonstrate the reactivity of the L4F3 antibody with in vitro hematopoietic colony-forming cells, including multipotential and unipotential progenitors.

Effects of L4F4 Plus C′ on Long-Term Marrow Cultures

Since treatment of marrow cells with L4F3 plus C′ lysed virtually all detectable immature colony-forming cells, we

Table 1. Generation of Colony-Forming Cells in Long-Term Culture System After Lysis With L4F3 + C′

		Colonies per Culture Tube					
		L4F3 + C′			T11D7 + C′		
Experiment	Week	CFU-GM	BFU-E*	CFU-Mix	CFU-GM	BFU-E*	CFU-Mix
I	0	217 ± 62 (92)†‡	0 ± 0 (>99)‡	0 ± 0 (>99)‡	3,779 ± 531	1,445 ± 266	118 ± 59
	1	12 ± 8‡	16 ± 8‡	0 ± 0‡	1,388 ± 165	419 ± 59	71 ± 18
	2	4 ± 4‡	0 ± 0‡	0 ± 0‡	1,447 ± 207	319 ± 59	65 ± 12
	3	176 ± 40	16 ± 16	20 ± 4	272‖	6‖	18‖
	4	216 ± 40§	60 ± 28	40 ± 20	434‖	59‖	41‖
	5	148 ± 16§	52 ± 12	28 ± 8	230‖	47‖	41‖
	6	108 ± 8	16 ± 4	8 ± 4	112‖	18‖	6‖
	7	49 ± 12	2 ± 1	13 ± 6	44‖	0‖	4‖
	8	40 ± 13	8 ± 4	8 ± 2	4‖	0‖	0‖
II	0	100 ± 200 (99)‡	300 ± 200 (95)‡	0 ± 0 (99)‡	8,400 ± 1,200	6,300 ± 900	1,100 ± 200
	1	540 ± 140‡	40 ± 60‡	20 ± 20‡	740 ± 80	560 ± 40	380 ± 20
	2	172 ± 16	96 ± 48	52 ± 24	148 ± 24	340 ± 224	44 ± 4
	3	256 ± 16	36 ± 20	40 ± 12	308 ± 32	124 ± 100	36 ± 16
	4	183 ± 6	23 ± 9	24 ± 14	20 ± 5	0 ± 0	0 ± 0
	5	192‖	16‖	16‖	0‖	0‖	0‖
III	0	190 ± 50 (96)‡	0 ± 0 (99)‡	0 ± 0 (99)‡	4,690 ± 480	3,500 ± 400	250 ± 50
	1	167 ± 27‡	504 ± 116	28 ± 5‡	803 ± 111	336‖	108‖
	2	1,142 ± 33	ND	ND	357 ± 147	ND	ND
	3	657 ± 82	20 ± 7	44 ± 33	550 ± 350	16‖	32‖
	4	765 ± 157	ND	ND	876 ± 141	ND	ND
	5	264‖	ND	ND	136‖	ND	

Abbreviation: ND, not determined

*Only bursts with two or more erythroid colonies were counted; small, single colonies were not included.

†Percent inhibition of colony growth at week 0 compared with control.

‡Number of colonies in cultures from L4F3-treated cells significantly less than from cultures of control-treated cells at $P < .01$

§Week 4, $.01 < P < .025$; week 5, $.025 < P < .05$

‖Results of a single culture plate

Equivalent numbers of marrow cells were treated with either L4F3 or T11D7 plus C′ and used to establish long-term cultures in the presence of irradiated (1,148 ± 207 cGy) 3- to 4-week-old marrow adherent cell layers. Based on pretreatment counts, 3×10^6 cells were cultured per tube in experiment I and 10^7 cells per tube in experiments II and III. Data are the means ± SEM of replicate cultures for each colony type. Total colonies per tube = (colonies/volume) × (volume in tube)/(volume removed)

examined the effect of L4F3 plus C′ on progenitor cells in long-term marrow cultures. Marrow cells treated with L4F3 plus C′ were cultured in the presence of irradiated confluent allogeneic adherent cell layers from 4-week-old long-term marrow cultures to provide a permissive environment for the proliferation of putative precursors of colony-forming cells.[34] No colony-forming cells were detectable in control cultures either of irradiated adherent cells alone or of irradiated cultures to which irradiated allogeneic marrow cells were added (data not shown).

In these experiments, equivalent numbers of marrow cells were treated with either L4F3 or T11D7 and C′, and the remaining cells, without adjustment for cell loss, were placed in long-term cultures and then assayed weekly for colony-forming cells. In representative experiments (Table 1), treatment with L4F3 plus C′ deleted greater than 92% of CFU-GM, 95% of BFU-E, and 99% of CFU-Mix when assayed immediately after treatment as compared with control antibody-treated cells. The cultures of L4F3 plus C′-treated cells showed significant and sustained increases in the numbers of CFU-GM, BFU-E, and CFU-Mix detectable in culture over time. After 2 to 3 weeks the numbers of these colony-forming cells (CFU-GM, BFU-E, CFU-Mix) detected in cultures of the L4F3-treated cells were similar to or greater than the numbers of colony-forming cells in the control cultures of T11D7-treated cells. Significantly fewer colony-forming

Table 2. Reactivity of Antibody L4F3 With Hematopoietic Progenitors: FACS

	Percentage of Total Population	CFU-GM	BFU-E	CFU-Meg	CFU-Mix
Unseparated	100	200.0 ± 2.1	56.7 ± 1.8	20.9 ± 1.0	5.0 ± 0.1
L4F3-positive	76	156.0 ± 2.7 (79)*	42.5 ± 1.1 (99)†	11.3 ± 0.4 (97)	2.6 ± 0.2 (100)
L4F3-negative	24	134.0 ± 3.6 (21)	0.8 ± 0.2 (1)	1.2 ± 0.4 (3)	0 ± 0

*Percentage of total colony-forming cells of each type present in sorted populations.

†BFU-E present in cultures of sorted cells were predominantly of the large, single-colony type. In cultures of unseparated cells, bursts with two or more colonies as well as single colonies were present

Separated and unseparated cells were cultured at 0.83×10^5/mL, and results are displayed as the mean ± SEM of colonies per 10^5 cells for five replicate cultures cells were detectable in cultures of L4F3-treated cells in the absence of irradiated marrow adherent cells as compared with those with adherent layers (data not shown).

Immediately following L4F3 treatment, BFU-E of two or more colonies were eliminated, and the only detectable erythroid progenitors formed almost exclusively small single colonies. Virtually all of the BFU-E subsequently detected in the long-term cultures of L4F3-treated cells consisted of two or more erythroid colonies, suggesting the origin of these bursts from less mature progenitors than were demonstrable at the initiation of cultures.[33,35] The CFU-Mix colonies detected in the long-term cultures of L4F3-treated cells were large, and the number detectable increased over the first 2 to 4 weeks of culture. These data suggest that precursors of immature BFU-E and CFU-Mix as well as CFU-GM do not express the L4F3 antigen.

Long-Term Cultures of L4F3-Stained Cells Separated by FACS

In cytolytic assays accessory cells in addition to colony-forming cells may be depleted. Therefore, we isolated L4F3-stained and unstained marrow cells using FACS. The resultant cells, placed in long-term cultures, were analyzed for CFU-GM production as an indicator of precursors of colony-forming cells since CFU-GM correlated with the production of other colony-forming cell types in our culture system. In a representative experiment (Table 3), L4F3-negative, L4F3-positive, and unseparated marrow cells were cultured over irradiated adherent cell layers. After 1 week, the number of CFU-GM detected in cultures of L4F3-negative cells was equivalent to or greater than the number in cultures of L4F3-positive cells despite the fact that the culture of L4F3-positive cells contained almost three times as many CFU-GM at initiation. Moreover, after 1 week, the number of CFU-GM in cultures of L4F3-negative cells was similar to that from cultures of unseparated cells. These results were corroborated in two additional experiments (data not shown). These data confirm that the precursors of colony-forming cells detectable after 3 weeks in long-term culture are not present in L4F3-positive marrow cell populations but are present in the L4F3-negative populations.

Table 3. Generation of CFU-GM in Long-Term Cultures From Isolated L4F3-Negative Cells

	Colonies per Culture Tube		
Weeks	L4F3-Negative	L4F3-Positive	Unseparated
Culture initiation	938 ± 56*	2,652 ± 102*	4,660 ± 117
1	1,344 ± 203	1,017 ± 102	1,127 ± 210
2	231 ± 34	429 ± 66	399 ± 57
3	760 ± 60	467 ± 17	809 ± 97
4	196†	8†	202†

*Mean ± SEM of CFU-GM per culture tube. Bone marrow cells were separated by FACS into negatively stained, positively stained, and unseparated populations The sorting window for positive cells contained 74% of the analyzed L4F3-stained marrow cells, and the negative window contained 25% of the marrow cells. Ambitubes with established marrow adherent cell layers were irradiated, and 7 × 10^5 of the L4F3-negative, 1 7 × 10^6 of the L4F3-positive, and 2.3 × 10^6 of the unseparated marrow cells were added to separate culture tubes. Single or duplicate cultures for CFU-GM were performed weekly from each individual tube. Colonies per tube = (colonies/volume removed) × (volume in culture tube)/(volume removed)

†Results of a single culture plate.

DISCUSSION

The present study extends our previous characterization of the L4F3 antigen expression by unipotent hematopoietic colony-forming cells to an assessment of multipotent colony-forming cells and, in long-term culture, the putative precursors of colony-forming cells. The L4F3 antigen has a relative molecular weight of 67,000 daltons and is also detected by the MY-9 antibody[24] as determined by competitive binding inhibition and comodulation studies (Andrews and Bernstein, unpublished observations, J. Griffin, personal communication). It is found at barely detectable levels on the surface of mature monocytes and granulocytes in immunofluorescence assays but in greater concentrations on immature granulocytic elements in bone marrow.[3] In the present study, we have shown by cytolysis and cell sorting that the L4F3 antigen is present on virtually all colony-forming cell types in bone marrow including CFU-GM, CFU-Meg, CFU-Mix, and immature BFU-E (two or more colonies/burst).

By culturing separated populations of marrow cells over irradiated allogeneic marrow adherent cell layers,[34] we have found that the L4F3 antigen is not expressed on the precursors of colony-forming cells. Thus, although L4F3 plus C′ treatment depleted virtually all immature colony-forming cells (CFU-GM, BFU-E, CFU-Mix), when the remaining cells were cultured over irradiated marrow adherent cell layers, increasing numbers of unipotent and multipotent colony-forming cells were detectable for up to 8 weeks. In the absence of an irradiated adherent cell layer, cultured L4F3-treated cells generated fewer colony-forming cells, and the cultures became senescent in 3 to 4 weeks. The data suggest that marrow cells treated with L4F3 produced more colony-forming cells for a longer time than do control cultures. Thus, it is possible that L4F3-expressing cells may inhibit the proliferation of these precursors of colony-forming cells.

The observation that cultures of L4F3-negative cells generated substantially greater numbers of colony-forming cells than were detectable at initiation strongly suggests that the L4F3-negative cells included precursors capable of substantial proliferative and/or differentiative activity in this culture system. Further, the length of time over which cultures contained detectable colony-forming cells, recent data on the cell cycle characteristics of committed colony-forming cells in long-term cultures,[35] and the cultures of L4F3-positive cells enriched for CFU-GM all suggest that it is unlikely that colony-forming cells detected in long-term cultures of L4F3-treated marrow represented the self renewal of a small number of cells committed to granulopoiesis.

Few other antigens present on immature multipotential as well as unipotential colony-forming cells have been described. Antibodies against HLA-DR determinants have been shown by a number of investigators to recognize CFU-GM, BFU-E, and CFU-Mix.[30,36–39] Non-HLA determinants include the antigen identified by antibodies 12.8,[40] MY-10,[25] and B1.3.C5,[41,42] which react with a 115,000-dalton antigen. The RFB-1 antibody described by Bodger et al[26] identifies an antigen expressed by CFU-E, BFU-E, CFU-GM and CFU-Mix as well as a subset of peripheral blood T lymphocytes. Ferrero et al[43] described a series of monoclonal antibodies reactive with progenitor cells; however, as in the case of RFB-1, the antigens identified by many of these antibodies have yet to be defined.

Less is known, however, about antigens expressed by the precursors of colony-forming cells. We have recently shown that the 12.8 antigen is expressed by the precursors of colony-forming cells detected in long-term cultures.[40] Ferrero et al[43] also have described antibodies reactive with pre–CFU-GM. Certain, though not all, HLA class II determinants are expressed on these cells,[30,38,39,44] and we now demonstrate that the L4F3 antigen is not expressed by these precursors.

Thus by using selection techniques as described in this paper, the precursors of colony-forming cells can now be separated from in vitro colony-forming cells and mature myeloid and lymphoid hematopoietic elements. The present studies suggest strategies for isolating these precursors that will make it possible to further characterize their requirements for proliferation and/or differentiation.

REFERENCES

1. Bernard A, Boumsell L, Bausset J, Milstein C, Schlossman SF (eds): Leucocyte Typing: Human Leucocyte Differentiation Antigens Detected by Monoclonal Antibodies. Berlin, Springer-Verlag, 1984

2 Bernstein ID, Self S. Joint report of the Myeloid Section of the Second International Workshop, in Reinherz E, Haynes BF, Nadler L, Bernstein ID (eds). Leucocyte Typing II, Vol 3. Berlin, Springer-Verlag, 1986, p 1

3 Andrews RG, Torok-Storb B, Bernstein ID· Myeloid associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies Blood 62:124, 1983

4. Bernstein ID, Andrews RG, Cohen SF, McMaster BE· Normal and malignant human myelocytic and monocytic cells identified by monoclonal antibodies J Immunol 128 876, 1982

5. Knapp W, Majdic O, Rumpold H, Bettelheim P, Kraft D, Liszka K, Forster O, Lutz D. Myeloid differentiation antigens as defined by monoclonal antibodies, in Knapp W (ed): Leukaemia Markers Orlando, Academic, 1981, p 207

6. Majdic O, Liszka K, Lutz D, Knapp W. Myeloid differentiation antigens defined by a monoclonal antibody. Blood 58.1127, 1981

7 Civin CI, Mirro J, Banquerigo ML· MY-1, a new myeloid-specific antigen identified by a mouse monoclonal antibody. Blood 57:842, 1981

8. Kemshead JT, Bicknell D, Greaves MF: A monoclonal antibody antibody detecting an antigen shared by neural and granulocytic cells. Pediatr Res 15:1282, 1981

9 Mannoni P, Janowska-Wieczorek A, Turner AR, McGamm L, Turc JM. Monoclonal antibodies against human granulocytes and myeloid differentiation antigens. Hum Immunol 5:309, 1982

10. Perussia B, Trinchieri G, Lebman D, Jankiewicz J, Lange B, Rovera G: Monoclonal antibodies that detect differentiation surface antigens on human myelomonocytic cells Blood 59:382, 1982

11. Skubitz KM, Pessano S, Bottero L, Perrero D, Rovera G, August JT. Human granulocyte surface molecules identified by murine monoclonal antibodies J Immunol 131.1882, 1983

12. Zola HY, McNamara P, Thomas M, Smart IJ, Bradley J The preparation and properties of monoclonal antibodies against human granulocyte membrane antigens Br J Haematol 48·481, 1981

13. Ball E, Granziano RF, Shen L, Fanger MW: Monoclonal antibodies to novel myeloid antigens reveal human neutrophil heterogeneity. Proc Natl Acad Sci USA 79:5374, 1982

14 Griffin JD, Ritz J, Nadler LM, Schlossman SF· Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 68:932, 1981

15 Todd RF, Nadler LM, Schlossman SF: Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126.1435, 1981

16. Linker-Israeli M, Billing RJ, Foon KA, Terasaki PI: Monoclonal antibodies reactive with acute myelogenous leukemia cells J Immunol 127:2473, 1981

17. Ugolini V, Nunez G, Smith RG, Stastny P, Capra JD: Initial characterization of monoclonal antibodies against human monocytes. Proc Natl Acad Sci USA 77:6764, 1980

18. Dimitriu-Bona A, Burmester GR, Waters SJ, Winchester RJ: Human mononuclear phagocyte differentiation antigens. I. Patterns of antigenic expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J Immunol 130·145, 1983

19 Anstee DJ, Edwards PAW: Monoclonal antibodies to human erythrocytes. Eur J Immunol 12:228, 1982

20. Sutherland R, Delia D, Schneider C, Newman R, Kemshead JT, Greaves M: Ubiquitous cell surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. Proc Natl Acad Sci USA 78:4515, 1981

21. McMichael AJ, Rust NA, Pilch R, Sochynsky R, Morton J, Mason DY, Ruan C, Tobelen G, Caen J: Monoclonal antibody to human platelet glycoprotein I. I. Immunological studies. Br J Haematol 49:501, 1981

22. McEver RP, Baenziger NL, Majerus PW: Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. J Clin Invest 66:1311, 1980

23. Vainchenker W, Deschamps JF, Bastin JM, Guichard J, Titeux M, Breton-Gorius J, McMichael AJ: Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: Immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients. Blood 59.514, 1982

24. Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8.521, 1984

25. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JF: Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133:157, 1984

26. Bodger M, Izaguirre CA, Blacklock HA, Hoffbrand AV. Surface antigenic determinants on human pluripotent and unipotent hematopoietic progenitor cells Blood 61:1006, 1983

27. Papayannopoulou T, Brice M, Yokochi T, Rabinovitch PS, Lindsley D, Stamatoyannopoulos G: Anti-HEL cell monoclonal antibodies recognize determinants that are also present in hemopoietic progenitors. Blood 63:326, 1984

28. Badin J, Levesque H. Precipitation rapide du facteur rhumatoide dans une solution d'acide borique et titrage pour l'agglutination des hematies humaines sensibilises. Rev Rhum Mal Osteoartic 28:101, 1961

29. Pesando JM, Tomaselli JK, Lazarus H, Schlossman SF: Distribution and modulation of a human leukemia-associated antigen (CALLA). J Immunol 131.2038, 1983

30. Keating A, Powell J, Takahashi M, Singer JW: The generation of human long-term marrow cultures from marrow depleted of Ia (HLA-DR) positive cells. Blood 64:1159, 1984

31. Powell JS, Fialkow PJ, Adamson JW: Human mixed cell colonies: Unicellular or multicellular origin-analysis by G-6-PD. Br J Haematol 57:89, 1984

32 Kimura H, Burstein S, Thorning D, Powell JS, Harker LA, Fialkow PJ, Adamson JW: Human megakaryocytic progenitors (CFU-M) assayed in methylcellulose: Physical characteristics and requirements for growth. J Cell Physiol 118:87, 1984

33. Eaves AC, Eaves CJ: Erythropoiesis in culture. Clin Haematol 13:371, 1984

34. Takahashi M, Keating A, Singer JW: A functional defect in irradiated adherent layers from chronic myelogenous leukemia long term marrow cultures. Exp Hematol 13:926, 1985

35. Cashman J, Eaves AC, Eaves CJ: Regulated proliferation of primitive hematopoietic progenitor cells in long-term human marrow cultures. Blood 66:1002, 1985

36. Fitchen JH, Ferrone S, Quaranto V, Molinaro GA, Cline MJ: Monoclonal antibodies to HLA-A,B and Ia-like antigens inhibit colony formation by human myeloid progenitor cells. J Immunol 125·2004, 1980

37 Fitchen JH, LeFevre C, Ferrone S, Cline MJ: Expression of Ia-like and HLA-A,B antigens on human multipotential hematopoietic progenitor cells. Blood 59:188, 1982

38. Moore MAS, Broxmeyer HE, Sheridan APC, Meyers PA, Jacobsen N, Winchester RJ: Continuous human bone marrow culture: Ia antigen characterization of probable pluripotent stem cells. Blood 55:682, 1980

39. Greenberger JS, Rothstein L, Defabritio P, Bregni M, Bast R, Ritz J, Nadler LM, Lipton JM, Sakakeeny MA: Effects of monoclonal antibody and complement treatment of human marrow on hematopoiesis in continuous bone marrow culture. Cancer Res 45:758, 1985

40. Andrews RG, Singer JW, Bernstein ID. Monoclonal antibody 12.8 recognizes a 115 Kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 67:842, 1986

41. Tindle RW, Nichols RAB, Chan L, Campana D, Catovsky D, Birnie GD: A novel monoclonal antibody B1-3C5 recognises myeloblasts and non-B non-T lymphoblasts in acute leukaemias and CGL blast crisis, and reacts with immature cells in normal marrow. Leuk Res 9:1, 1985

42. Katz FE, Tindle RW, Sutherland R, Greaves MF: Reactivity of a monoclonal antibody defining human haemopoietic multipotential progenitors, in Reinherz E, Haynes B, Nadler LM, Bernstein ID: Leucocyte Typing II, vol 3. New York, Springer-Verlag, 1986, p 305

43. Ferrero D, Gabbianelli M, Peshle C, Lange B, Rovera G: Surface phenotypes of human hemopoietic progenitor cells defined by monoclonal antibodies Blood 66:496, 1985

44. Falkenberg JHF, Fikke WE, Goselink HM, van Rood JJ, Jansen J: Human hematopoietic progenitor cells in long-term cultures express HLA-DR antigens and lack HLA-DQ antigens J Exp Med 162.1359, 1985

Bromodeoxyuridine Treatment of Normal Adult Erythroid Colonies: An In Vitro Model for Reactivation of Human Fetal Globin Genes

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We report that bromodeoxyuridine (BrdU) addition in semisolid cultures of normal adult erythroid progenitors causes a sharp rise of γ-globin gene expression in erythroid colonies. Control studies were carefully carried out to exclude the possibility of toxic effects exerted by the drug in these experimental conditions. In particular, BrdU addition induces a sharp increase in the level of relative γ-globin synthesis and content in pooled BFU-E–derived colonies: this rise is clearly observed in single bursts of the mature type (largely composed of late erythroblasts) but not in immature ones (essentially comprising early erythroblasts). Furthermore, it is associated with an increase of the $^G\gamma$ / $^G\gamma$ + $^A\gamma$ synthetic ratio from adult up to fetal like values. Reactivation of γ-synthesis was observed even if BrdU was added to colonies composed essentially of early erythroblasts, ie, when BrdU was added to either bursts at day 10 of culture or late CFU-E–derived clones at day 1.

These in vitro observations indicate modulation of γ-synthesis at the stage of erythroblasts from normal adults. At the molecular level we suggest that BrdU, by replacing thymidine in DNA, may inhibit the switch from a fetallike biosynthetic program expressed in early erythroblastic differentiation to the adult program expressed in later stages of maturation.

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IN ADULT HUMANS, fetal hemoglobin (HbF, $\alpha_2\ \gamma_2$) represents <1% of total Hb, the remainder being adult Hb (essentially HbA, $\alpha_2\ \beta_2$). Nevertheless, a significant potential for HbF synthesis is retained by normal adult erythroid progenitors, not only by early ones (BFU-E)[1–4] but also by late ones (CFU-E).[5–7] This was first shown in vitro, on the basis of the enhanced HbF content or synthesis in erythropoietic colonies generated in culture in BFU-E[1–4] and CFU-E.[5–7]

Recently, De Simone et al[8] reported that anemic baboons show a marked rise of HbF levels after treatment with 5-azacytidine (5-azaC), ie, a nucleoside analogue known to inhibit DNA methylation in vivo. Similar results were observed with other antiblastic agents such as vinblastine[9] and hydroxyurea.[10] More important, a striking increase in HbF levels has been observed in patients with different hemoglobinopathies (ie, sickle cell diseases and β-thalassemia) treated with 5-azaC.[11–14] These studies have generated considerable debate[15,16] because they represent one of the first attempts to treat a human hereditary disease by pharmacologic manipulation of the expression of specific genes. Furthermore, they reflect on the debated issue of mechanisms controlling HbF synthesis in adult life, which may admittedly act at the level of erythroid progenitors[1–4] and/or immature erythroblasts.[5,17,18]

In an attempt to establish an in vitro approach to these aspects, we have analyzed the globin chains' synthesis pattern in normal human adult erythroid colonies treated with 5-azaC, bromodeoxyuridine (BrdU), and other agents at various stages of their erythropoietic differentiation We report here that BrdU treatment of normal adult erythroid bursts causes a striking increase in their relative fetal globin synthesis and content, at least partially via a direct effect at the level of differentiating erythroblasts.

MATERIALS AND METHODS

Culture of Erythroid Colonies

Culture of erythroid bursts. Heparinized blood was obtained from the antecubital vein of normal adult volunteers. The mononuclear cells, harvested by a slight modification[19] of the Ficoll-Isopaque technique,[20] were grown in methylcellulose cultures as described.[21–22] Each 1-mL plate contained the following components in Iscove's modified Dulbecco's medium (IMDM): methylcellulose (0.8%, final concentration), α-thioglycerol (10^{-4} mol/L), fetal calf serum (FCS) (30%), 3×10^5 nucleated cells, and semipurified erythropoietin (2 IU, kindly provided by the National Heart, Lung and Blood Institute, 1,140 IU/mg of protein) The plates, carefully maintained in the dark, were incubated in a humidified 5% CO_2 air atmosphere at 37 °C. BrdU (Sigma Chemical Co, St Louis) dissolved in IMDM was added to the dishes at sequential days of culture (ie, day 0, 6, 8, or 10). An equivalent volume of IMDM was supplemented in control plates. On day 12, erythroid bursts were recognized in situ on the basis of their pink-orange-red color and further classified as will be described

Classification of erythroid bursts. Maturation of BFU-E–derived bursts is not only gradual but also asynchronous: the asynchrony is clearly observed when comparing different bursts in a culture dish. Since the relative level of γ-chain synthesis in bursts depends upon their stage of maturation,[18] it seemed necessary to classify each burst according to its maturation stage. Thus, day 12 erythroid bursts were classified in (1) white-pink immature colonies (mainly composed of proerythroblasts and basophilic erythroblasts) and (2) orange-red mature clones (largely composed of polychromatic and orthochromatic erythroblasts). Control studies involving pickup of single colonies and May-Grünwald staining of the cytocentrifuged smears documented that immature or mature bursts comprised ≤20% to 30% or ≥30% to 50% polychromatophilic-orthochromatic erythroblasts respectively.

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Culture of day 4 CFU-E clones. Bone marrow specimens were obtained from hematology-oncology patients without marrow involvement prior to initiation of therapy. Monocellular marrow suspensions obtained as described[21] were enriched with immature erythroblasts and progenitors by standard procedures, ie, two sequential density cuts (Ficoll-Hypaque, Pharmacia Fine Chemicals, Uppsala, Sweden) and then two rounds of adherent cell removal in plastic flasks Cells were cultured in methylcellulose as described before (10^5 enriched marrow cells/mL). BrdU (1 μg/mL) was added on day 1 CFU-E clones (50 to 100 cells/colony) were labeled on day 3 and scored on day 4

Radioactive Labeling of Globin Chains in Erythroid Colonies

Erythroid bursts On day 12 of culture immature and mature bursts were recognized in situ, randomly picked up with a fine Pasteur pipette in 2- to 3-μL volume,[23] and labeled, either individually or in pools of five colonies, in 10 μL of leucine-free RPMI 1640 medium containing 25 to 50 μCi of ^{3}H-leucine (specific activity, >100 Ci/mmol/L, Amersham, Great Britain) as described.[21,22] After a 16- to 24-hour incubation period, the colonies were washed in 1 2 mL of cold saline (containing 5 × 10^5 carrier RBC) and stored at −30 °C. Globin synthesis was analyzed as will be indicated.

Culture of day 4 CFU-E–derived clones ^{3}H-leucine (100 μCi/plate) was added at day 3 of culture Colonies were picked up by means of a thin Pasteur pipette and their pool, derived from three separate dishes, analyzed for globin chain synthesis.

Control Experiments for BrdU cytotoxicity. These experiments were carried out to verify the possible cytotoxic effect induced by BrdU on the growth of erythroid bursts This agent was added at the various culture times and concentrations used here (see Results). Only IMDM was plated in control dishes. For each experimental point (comprising at least three replicate dishes), immature and mature bursts were scored and then separately pooled, washed, and resuspended in 1 mL of RPMI The number of viable cells (>95%) was evaluated by the standard trypan blue technique (Flow Laboratories, Great Britain) and their morphology analyzed on cytocentrifuged smears stained with May-Grünwald plus Giemsa. Each pool of cells was subdivided in two aliquots. The first one was analyzed for its total Hb content according to methods described.[24] The second one was centrifuged and resuspended in 100 μL of RPMI containing 100 μCi of ^{3}H-leucine (for further details see the preceding material): globin synthesis and content were then analyzed in the same gel, as will be indicated.

These studies allowed the evaluation of the following parameters: number of bursts per dish and of cells per burst, morphology of cells in each pool, Hb content per cell, and globin chain synthesis and content in each pool.

Globin preparation and analysis. Globin prepared from stroma-free lysates by the acid acetone procedure was analyzed by means of isoelectric focusing (IEF) in the presence of Nonidet P-40 according to the methods described previously.[23,25] Gel fluorography and scanning of the exposed film were performed as described.[22]

In some experiments, synthesis and content of globin chains was evaluated on the same gel: the latter parameter was analyzed by staining according to the Coomassie brillant blue method (Bio-Rad Laboratories, Richmond, Calif) followed by gel fluorography.

RESULTS

In the first series of experiments mononuclear cells from peripheral blood of normal adult volunteers were plated in methylcellulose culture and erythroid bursts grown and scored under standard conditions in the presence of various BrdU concentrations (0.3 to 30 μmol/L). When added on days 0 to 6 of culture, BrdU induces a dose-dependent decrease of the number of erythroid bursts scored on days 12 and 13 as compared with untreated controls (Fig 1). However, addition of 0.3 to 3.0 μmol/L BrdU on days 8 and 10 causes little or no inhibition as confirmed by analysis of variance (Fig 1). The relative synthesis of β-, $^G\gamma$-, and $^A\gamma$-globin was determined on day 12 by means of IEF of in vitro labeled ^{3}H-globin. Control $\gamma/\ \gamma + \beta$ synthetic ratios ranged from 0.10 to 0.30 depending upon the maturation degree of the analyzed colonies and the FCS batch used.[18] A marked increase of fetal globin production was observed at all tested BrdU concentrations for both early (day 6) and late addition times (days 8 to 10) (results not presented here).

These experiments, although of potential interest, emphasized the necessity to exclude the possibility that the action of BrdU on γ-synthesis was due to toxic effects induced by this agent on erythropoietic cells growing in vitro. Therefore, a second series of studies was initiated to establish the experimental conditions allowing BrdU addition to culture without detectable toxic effects on erythroid proliferation and differentiation. As shown in Table 1, addition of BrdU at low doses (0.3 to 3.0 μmol/L) and late addition times (days 8 or 10 in culture) did not modify the growth and differentiation of pooled erythroid bursts of the mature type as compared with control colonies. Equivalent results were obtained for immature bursts pooled from the same plates. The evaluated parameters included the number of colonies per plate and cells per colony as well as the mean Hb content in each cell. Lack of variation in the colony number rendered unlikely the possibility that BrdU addition causes recruitment of a new cohort of BFU-E for differentiation in culture. It is also of interest that BrdU did not significantly modify the number of cells per colony, the mean Hb content per cell, as well as the efficiency of radioactivity incorporation (not shown) (as

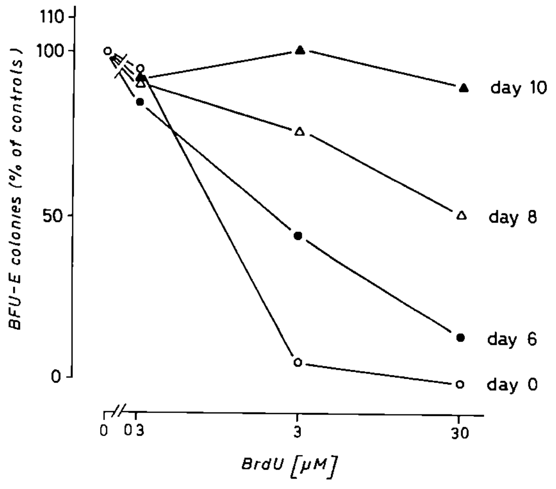

Fig 1. Effect of BrdU addition at sequential days of culture on the growth of normal adult BFU-E colonies. The mean number of colonies from four different experiments is presented (100% value, 105.6 ± 7.8 (mean ± SD) colonies/ 3 × 10^6 light density blood cells). Analysis of variance indicated no statistical difference between the number of BFU-E colonies treated with 0.3 to 3.0 μmol/L BrdU on days 8 to 10 and control values.

Table 1. BrdU Addition at Days 8 to 10 of Culture: Effect on BFU-E Cloning Efficiency and Hb Content per Cell on Day 12

BrdU Addition (Day and Dose)	Burst Type	No. of Bursts per Dish	No of Cells per Burst (× 10^3)	Hb Content per Cell (pg)
Controls	Immature	44	6.1	10.8
	Mature	62.3 ± 3.3	10.0 ± 2.4	24.4 ± 1.5
Day 8				
0.3 µmol/L	Immature	42	6.0	10.6
	Mature	57.3 ± 4.7	9.4 ± 2.4	22.6 ± 5.6
3.0 µmol/L	Immature	33.1	ND	ND
	Mature	48.3 ± 3.0	6.0 ± 1.0*	25.6 ± 2.0
Day 10				
0.3 µmol/L	Immature	34	7.5	12.3
	Mature	66.0 ± 1.3	10.3 ± 3.4	27.0 ± 3.2
3.0 µmol/L	Immature	53	6.2	10.0
	Mature	57.5 ± 8.4	10.6 ± 1.4	24.4 ± 4.2

Abbreviation: ND, not determined.

*$P < .05$ when compared with control values.

Mean ± SEM values from three separate experiments for mature bursts. Only one experiment was performed for immature bursts. For further details see the text.

a single exception, the number of cells per burst upon addition of 3.0 µmol/L BrdU on day 8 is slightly but significantly diminished when compared with control values, $P < .05$). In conclusion, these control studies excluded the possibility that BrdU addition, at low doses (0.3 to 3.0 µmol/L) and late culture times (days 8 or 10), may exert toxic effects on erythroblasts, hampering their proliferative rate and/or their biochemical machinery for globin chain production.

In a third series of experiments on globin synthesis, BrdU was therefore added only at late culture times (days 8 to 10), particularly at low doses (0.3 to 3.0 µmol/L), thus precluding the possible impact of erythroid cell toxicity. Representative results are summarized in Fig 2. In pooled bursts from two different volunteers (nos. 8 and 44) comprising both immature and mature colonies, BrdU addition induces not only an approximate twofold increase of the relative proportion of fetal globin synthesis (0.24 → 0.43 and 0.32 → 0.55 respectively), but also a significant rise of the ${}^{G}\gamma$ / ${}^{G}\gamma + {}^{A}\gamma$ globin ratio (from 0.56 up to 0.66) (Fig 2A). The elevation of both parameters is more marked when evaluating selectively the pool of mature bursts (Fig 2B, volunteer 44), also resulting from the lower level in control mature colonies as compared with the pool of all bursts. In particular, the ${}^{G}\gamma$ / ${}^{G}\gamma + {}^{A}\gamma$ ratio rises from a typical adult value of 0.40 to a fetallike level of 0.50 to 0.60.[22]

It seemed also of interest to comparatively evaluate synthesis and content of globin chains in pooled BrdU-treated *v* control bursts of the mature type. As shown in Table 2, BrdU treatment induced a twofold increase of both relative γ-chain synthesis level, and of the ${}^{G}\gamma$ / ${}^{G}\gamma + {}^{A}\gamma$ ratio. In these experiments we have also evaluated the parameters assayed in the control studies mentioned before (number of bursts per dish and cells per burst, Hb content per cell, and morphology of cells in pooled bursts): the observed values were superimposable with those in Table 1 (not shown).

In the fourth series of experiments, single bursts at different stages of hemoglobinization (ie, either immature or mature) were individually labeled and analyzed: a marked rise of both the γ / $\gamma + \beta$ and ${}^{G}\gamma$ / ${}^{G}\gamma + {}^{A}\gamma$ ratios is observed only for mature colonies (Fig 3A and B). It is emphasized that BrdU was added on day 10 of culture, whereas the bursts were labeled as always on day 12. Since bursts matured on day 12 are essentially composed of early erythroblasts on day 10, the rise of the relative γ-chain synthesis in mature day 12 bursts may be explained by the action of BrdU on erythroblasts that are in these colonies on days 10 to 12.

Finally, the effect of BrdU addition (3 µmol/L at day 1 of culture) on Hb synthesis has been evaluated in pools of

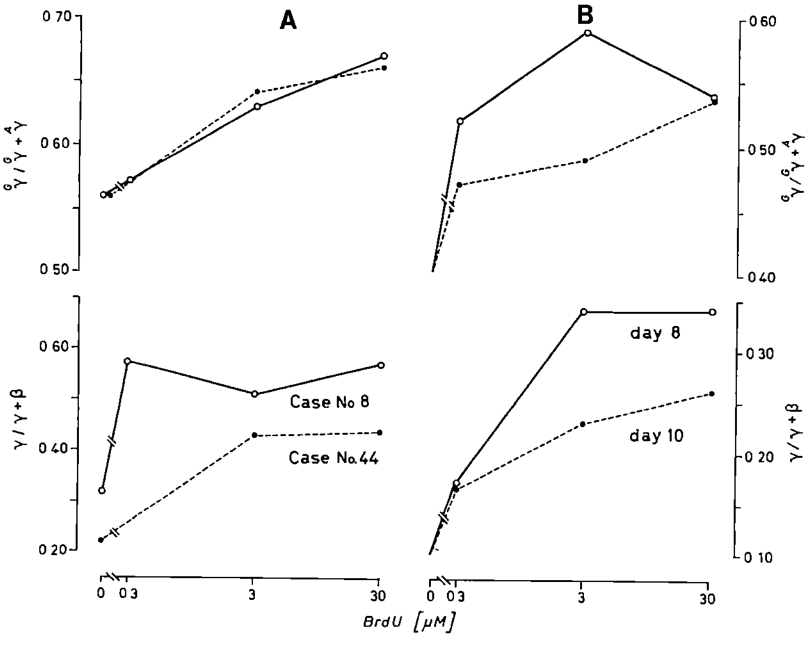

Fig 2. Effect of BrdU addition on ${}^{G}\gamma/{}^{G}\gamma + {}^{A}\gamma$ and $\gamma/\gamma + \beta$ synthetic ratios in (A) pooled (immature plus mature) bursts from two different normal volunteers (nos. 8 and 44) (left) and (B) pooled mature bursts from volunteer 44 (right). BrdU was added on days 8 (A) or 8 and 10 (B). Each point represents the mean value from at least three different pools. Analysis of variance showed that all the BrdU-treated values significantly differ from corresponding controls (except for ${}^{G}\gamma/{}^{G}\gamma + {}^{A}\gamma$ values at 0.3 µmol/L values in pooled bursts). For further explanations, see text.

Table 2. Comparative Analysis of the Effect of BrdU Addition on Globin Chain Content and Synthesis in Mature Bursts

	Content		Synthesis	
Treatment	$\gamma/\gamma+\beta$	$^{G}\gamma/^{G}\gamma+{}^{A}\gamma$	$\gamma/\gamma+\beta$	$^{G}\gamma/^{G}\gamma+{}^{A}\gamma$
Control	0.38	0.26	0.26	0.33
BrdU (3 μmol/L on day 8)	0.69	0.51	0.48	0.53

See also the legend to Fig 3.

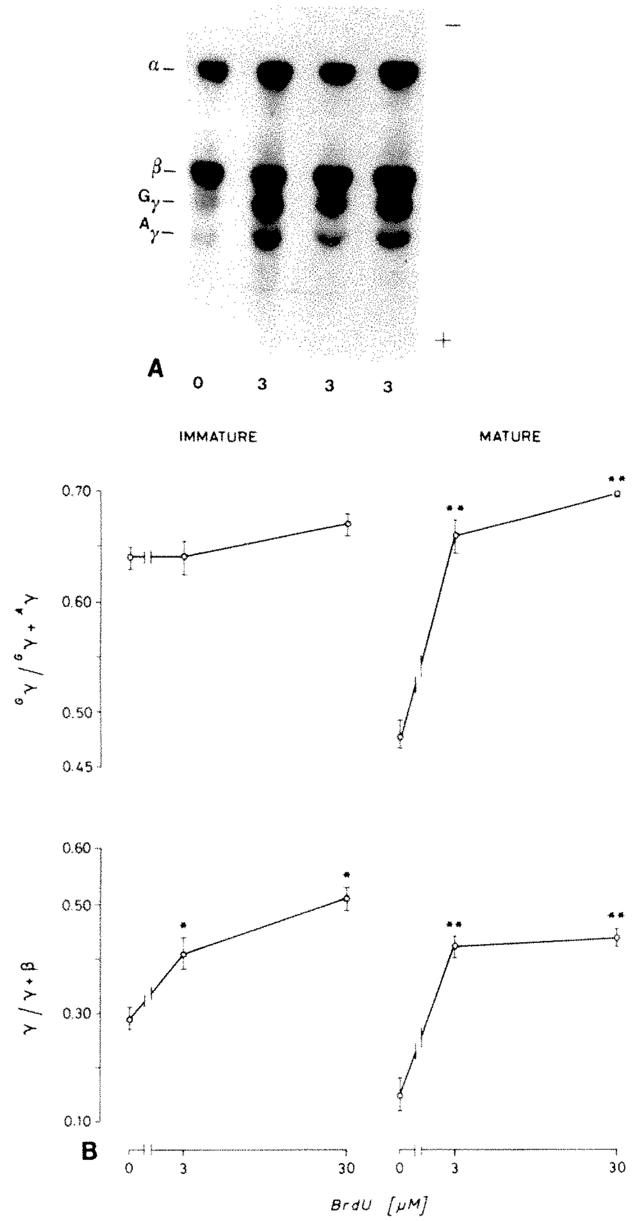

Fig 3. (A) Fluorogram of the IEF pattern of single mature bursts treated with BrdU on day 10 and labeled on day 12. Control bursts (0) are also presented. (B) Effect of BrdU addition of $^{G}\gamma/^{G}\gamma+{}^{A}\gamma$ and $\gamma/\gamma+\beta$ synthetic ratios in single immature or mature colonies treated as above. Mean ± SEM values are presented. Each point represents a minimum of five bursts. Key: *, $P<.001$; **, $P<.0001$ as compared with control values.

erythroid colonies generated by day 4 CFU-E (also termed high-Ep–sensitive cells), which are closely related to proerythroblasts.[26] Once again, a twofold increase of the $\gamma/(\gamma+\beta)$ synthetic ratio has been observed in BrdU-treated clones as compared with control values (0.34 and 0.16 respectively), thus confirming that BrdU modulates the γ-chain synthesis in vitro at the erythroblast level.

DISCUSSION

Reactivation of γ-globin genes in adult life is currently subject to intensive investigation. In this regard, various antiblastic drugs (5-azaC, hydroxyurea, vinblastine, etc) appear to increase HbF levels in vivo.[9-14] The present experiments report an in vitro model for this phenomenon based on the action of BrdU on erythroid colonies generated by normal adult BFU-E and day 4 CFU-E.

Mechanisms underlying the in vivo action of antiblastic agents, particularly 5-azaC, are still a matter of controversy.[15-16] Hypomethylation is generally linked to derepression of eukaryotic genes[27,28]: expression of ϵ and γ genes in purified embryonic or fetal erythroblasts is directly correlated with undermethylation in the corresponding domains, particularly in their 5′ flanking region.[29] It is debatable, however, whether 5-azaC acts by preventing the methylation of cytosine in the DNA. In β-thalassemic patients treated with 5-azaC, both the embryonic ϵ- and the fetal γ-globin gene domains are undermethylated, but only the latter is activated.[11] On the other hand, 5-azaC–treated sickle cell anemia patients show a selective undermethylation of the 5′ flanking region of γ genes that is associated with enhanced γ-globin synthesis.[13] In view of these uncertainties, the alternative possibility was suggested that this cytotoxic, cell cycle–specific drug might preferentially kill rapidly dividing erythroid colony-forming units (CFU-E), thus recruiting earlier progenitors (BFU-E)[30] that retain an elevated HbF-synthetic potential.[1-4] This hypothesis, however, is not easily reconciled with in vitro experiments showing that normal adult CFU-E incubated for 24 hours with 5-azaC give rise to colonies with increased HbF content.[14] Furthermore, sickle cell anemia and severe thalassemia patients administered with 5-azaC often show a very rapid rise of peripheral F cells as early as 24 to 48 hours after treatment[14,31]: this suggests but does not prove that the drug may also exert its action via a direct erythroblastic mechanism.

In our in vitro experiments, BrdU cytotoxicity has been carefully excluded by appropriate control studies. Furthermore, the relative γ-globin synthesis and content is markedly enhanced in bursts matured on day 12 even if BrdU had been added as late as day 10 (ie, at a time when these colonies comprised essentially immature erythroblasts). Along these lines, addition of BrdU at day 1 of culture induces a significant rise of the relative γ-chain synthesis in day 4 erythroid colonies generated by late CFU-E, which correspond to proerythroblasts.[26] It is hence concluded that, at least in these experiments, BrdU exerted its action essentially at the level of erythroblasts, particularly of immature type, whereas a major effect on early undifferentiated progenitors (BFU-E, day 7 CFU-E) is seemingly excluded.

These in vitro observations represent the first direct evidence of modulation of γ-synthesis in erythroblasts from normal adults. They clearly shed light on a model of regulation of HbF production in adult life and imply that modulation at the erythroblastic level may be a major control mechanism as previously postulated by our group.[5,17,18]

At the molecular level, an intriguing interpretation of the BrdU effect on differentiating cellular systems was suggested by Weintraub et al.[32] BrdU incorporation in DNA at the place of thymidine might affect specific targets corresponding to genes involved in specifying new developmental programs (switching loci) and would therefore inhibit initiation of these programs. There is a clear analogy with our system because it is known that the peak of HbF synthesis occurs earlier than that of HbA in both adult[33] and fetal[34] erythroblast maturation: this implies a switch from an early to a late program for globin gene expression in the erythroid maturation pathway. It is then highly significant that BrdU increases HbF synthesis in mature but not in immature bursts. Furthermore, this effect is accompanied by an augmented $^G\gamma / ^G\gamma + {}^A\gamma$ synthetic ratio, but only in mature colonies. We tentatively conclude that BrdU prevents the switch from a fetallike biosynthetic program in early erythroblastic differentiation to the adult program expressed in later stages of maturation.

As mentioned before, alternative interpretations seem unlikely. Indeed, the BrdU action on γ-synthesis can hardly be attributed to a mitotic arrest at the early erythroblastic level since the number of erythroid cells per colony was not modified by addition of the drug. At the molecular level, a blockade of the biochemical machinery for globin chain synthesis by BrdU is seemingly excluded, since both the mean Hb content per cell and the efficiency of radioactivity incorporation are not modified by the drug. If our interpretation is correct, further studies with labeled BrdU may allow the pinpointing of critical DNA regions involved in the globin gene expression switch during erythroid maturation, and also hypothetically in the analogous ontogenic Hb switch in perinatal life.

It should be emphasized that our interpretation of the BrdU results does not necessarily reflect the increased HbF production induced by other antiblastic agents, particularly 5-azaC. In this regard, 5-azaC induces activation of a variety of genes in numerous cell systems,[35] whereas BrdU provokes a blockade of gene activation[32] as postulated in these studies.

In conclusion, the in vitro model with BrdU addition suggests a drug-sensitive step in γ-globin synthesis during the early phase of differentiation of normal adult erythroblasts. It may hence contribute to future studies designed to pharmacologically manipulate globin gene expression, as well as shed light on mechanisms controlling HbF expression in normal adult life.

REFERENCES

1. Papayannopoulou Th, Brice M, Stamatoyannopoulos G: Hemoglobin F synthesis in vitro: Evidence for control at the level of primitive erythroid stem cells. Proc Natl Acad Sci USA 74:2923, 1977

2. Kidoguchi K, Ogawa M, Karam JD: Hemoglobin biosynthesis in individual erythropoietic bursts in culture. J Clin Invest 63:804, 1979

3. Peschle C, Migliaccio G, Covelli A, Lettieri F, Migliaccio AR, Condorelli M, Comi P, Pozzoli ML, Giglioni B, Ottolenghi S, Cappellini MD, Polli E, Gianni AM: Hemoglobin synthesis in individual bursts from normal and adult blood: All bursts and subcolonies synthesize $^G\gamma$- and $^A\gamma$-globin chains. Blood 56:218, 1980

4. Stamatoyannopoulos G, Papayannopoulou Th, Brice M, Kurachi S, Nakamoto B, Lim G, Farquhar M: Cell biology of hemoglobin switching I. The switch from fetal to adult hemoglobin formation during ontogeny, in Stamatoyannopoulos G, Nienhuis AW (eds): Hemoglobins in Development and Differentiation. Alan R. Liss, New York, Liss, 1981, p 287

5. Comi P, Giglioni B, Pozzoli ML, Ottolenghi S, Gianni AM, Migliaccio AR, Migliaccio G, Lettieri F, Peschle C: Biosynthesis of globin chains in fetal liver and adult marrow cultures. Comparative analysis of individual colonies derived from early, intermediate or late erythroid progenitors. Exp Cell Res 133:347, 1981

6. Dover GJ, Chan T, Sieber F: Fetal hemoglobin production in cultures of primitive and mature human erythroid progenitors: Differentiation affects the quantity of fetal hemoglobin produced per fetal-hemoglobin–containing cell. Blood 61:1242, 1983

7. Friedman AD, Linch DC, Miller B, Lipton JM, Javid T, Nathan DG: Determination of the hemoglobin F program in human progenitor-derived erythroid cells. J Clin Invest 75:1359, 1985

8. De Simone J, Heller P, Hall L, Zwiers D: 5-Azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. Proc Natl Acad Sci USA 79:4428, 1982

9. Veith R, Papayannopoulou Th, Kurachi S, Stamatoyannopoulos G: Treatment of baboon with vinblastine: Insights into the mechanisms of pharmacologic stimulation of HbF in the adult. Blood 66:456, 1985

10. Letvin NL, Linch DC, Bardsley P, McIntire KW, Nathan DG: Augmentation of fetal hemoglobin production in anemic monkeys by hydroxyurea. N Engl J Med 310:869, 1984

11. Ley TJ, De Simone J, Anagnou NP, Keller GH, Humphries RK, Turner PH, Young NS, Heller P, Nienhuis AW: 5-azacytidine selectively increases γ-globin synthesis in a patient with β-thalassemia. N Engl J Med 307:1469, 1982

12. Ley TJ, De Simone J, Naguchi CT, Turner PH, Schechter AN, Heller P, Nienhuis AW: 5-azacytidine increases γ-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. Blood 62:370, 1983

13. Charache S, Dover G, Smith K, Talbot CC Jr, Moyer M, Boyer S: Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the γ-δ-β complex. Proc Natl Acad Sci USA 80:4842, 1983

14. Humphries RK, Dover G, Young NS, Moore JG, Charache S, Ley T, Nienhuis AW: 5-azacytidine acts directly on both erythroid precursors and progenitors to increase production of fetal hemoglobin. J Clin Invest 75:547, 1985

15. Orkin SH: Controlling the fetal globin switch in man. Nature 301:108, 1983

16. Kolata G: Globin gene studies create a puzzle. Science 223:470, 1984

17. Peschle C, Migliaccio AR, Migliaccio G, Lettieri F, Maguire YP, Condorelli M, Gianni AM, Ottolenghi S, Giglioni B, Pozzoli ML, Comi P: Regulation of Hb synthesis in ontogenesis and erythropoietic differentiation. In vitro studies on fetal liver, cord blood, adult blood or marrow, and in HPFH patients, in Stamatoyannopoulos G, Nienhuis AW (eds): Hemoglobin in Development and Differentiation. New York, Liss, 1981, p 359

18. Peschle C, Migliaccio G, Migliaccio AR, Covelli A, Giuliani A, Mavilio F, Mastroberardino G: Hemoglobin switching in humans, in Dunn C (ed): Current Concepts in Erythropoiesis. London, Wiley, 1983, p 339

19. Ogawa M, Grush OC, O'Dell RR, Hara H, MacEarn MD: Circulating erythropoietic precursors assessed in culture: Characterization in normal man and patients with hemoglobinopathies. Blood 50:1081, 1977

20. Böyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Invest 21:7, 1968 (suppl 97)

21. Peschle C, Migliaccio AR, Migliaccio G, Lettieri F, Quattrin S, Russo G, Mastroberardino G: Identification and characterization of three classes of erythroid progenitors in fetal liver. Blood 58:565, 1981

22. Peschle C, Migliaccio AR, Migliaccio G, Petrini M, Calandrini M, Russo G, Mastroberardino G, Presta M, Gianni AM, Comi P, Giglioni B, Ottolenghi S: The embryonic → fetal Hb switch in humans: Studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver. Proc Natl Acad Sci USA 81:2416, 1984

23. Comi P, Giglioni B, Ottolenghi S, Polli E, Gianni AM, Barba P, Covelli A, Migliaccio G, Condorelli M, Peschle C: Globin chain synthesis in single erythroid bursts from cord blood: Studies on $\gamma \rightarrow \beta$ and ${}^{G}\gamma \rightarrow {}^{A}\gamma$ switches. Proc Natl Acad Sci USA 77:362, 1980

24. Conscience JF, Miller RA, Henri J, Ruddle FH: Acetylcholinesterase carbonic anhydrase and catalase activity in Friend erythroleukemic cells, non-erythroid mouse cell lines and their somatic hybrids. Exp Cell Res 105:401, 1977

25. Comi P, Giglioni B, Ottolenghi S, Gianni AM, Ricco G, Mazza U, Saglio G, Camaschella C, Pich PG, Gianazza E, Righetti PG: ${}^{G}\gamma$ and ${}^{A}\gamma$ globin chains separation and quantitation by isoelectric focusing. Biochem Biophys Res Commun 87:1, 1979

26. Alter BP, Gilbert HS: Hydroxyurea and hemoglobin F in patients with myeloproliferative syndromes, in Stamatoyannopoulos G, Nienhuis AW (eds): Experimental Approaches for the Study of Hemoglobin Switching. New York, Liss, 1985, p 455

27. Razin A, Riggs AD: DNA methylation and gene function. Science 210:604, 1980

28. Felsenfeld G, McGhee J: Methylation and gene control. Nature 296:602, 1980

29. Mavilio F, Giampaolo A, Carè A, Migliaccio G, Calandrini M, Russo G, Pagliardi GL, Mastroberardino G, Marinucci M, Peschle C: Molecular mechanisms of human hemoglobin switching: Selective undermethylation and expression of globin genes in embryonic, fetal and adult erythroblasts. Proc Natl Acad Sci USA 80:6907, 1983

30. Torrealba de Ron AT, Papayannopoulou Th, Knapp MS, Feng Ruen Fu M, Knitter G, Stamatoyannopoulos G: Perturbations in the erythroid marrow progenitor cell pools may play a role in the augmentation of HbF by 5-azacytidine. Blood 63:201, 1984

31. Dover GJ, Charache SH, Boyer SH, Conover Talbot C Jr, Smith KD: 5-Azacytidine increases fetal hemoglobin production in a patient with sickle cell disease, in Stamatoyannopoulos G, Nienhuis AW (eds): Globin Gene Expression and Hematopoietic Differentiation, New York, Liss, 1983, p 475

32. Weintraub H, Campbell GL, Holtzer H: Differentiation in the presence of bromodeoxyuridine is "all-or-none." Nature 244:140, 1973

33. Papayannopoulou T, Kalamantis T, Stamatoyannopoulos G: Cellular regulation of hemoglobin switching: Evidence for inverse relationship between fetal hemoglobin synthesis and degree of maturity of human erythroid cells. Proc Natl Acad Sci USA 76:6420, 1979

34. Gianni AM, Comi P, Giglioni B, Ottolenghi S, Migliaccio AR, Migliaccio G, Lettieri F, Maguire YP, Peschle C: Biosynthesis of Hb in individual fetal liver bursts: γ-Chain production peaks earlier than β-chain in the erythropoietic pathway. Exp Cell Res 130:345, 1980

35. Mohandas T, Sparkes RS, Shapiro LJ: Reactivation of an inactive human X chromosome: Evidence of X inactivation by DNA methylation. Science 211:393, 1981

Childhood Ki-1 Lymphoma Presenting With Skin Lesions and Peripheral Lymphadenopathy

By Marshall E. Kadin, Dianne Sako, Nancy Berliner, Wilbur Franklin, Bruce Woda, Michael Borowitz, Karen Ireland, Abe Schweid, Philip Herzog, Beverly Lange, and Ronald Dorfman

We describe a large-cell lymphoma of activated lymphoid cells in six children and adolescents. The presenting clinical features of regressing skin lesions and peripheral lymphadenopathy, sinus infiltration of lymph nodes, and infrequent tumor cell erythrophagocytosis resulted in initial diagnoses of malignant or regressing atypical histiocytosis in five cases. Binucleate and multinucleate tumor cells, sometimes with prominent eosinophilic nucleoli, resembled Reed-Sternberg (RS) cells and occasionally were found in a cytoarchitectural milieu that was consistent with a diagnosis of Hodgkin's disease (HD). The tumor cells did in fact express the HD-associated antigen Ki-1, but unlike most types of HD, the RS-like cells expressed common leukocyte antigen and were negative for Leu-M1. A T cell origin for the malignant cells was demonstrated with monoclonal antibodies in two cases, by focal staining for nonspecific esterase in one case, and by rearrangement of the β-chain genes for the T cell receptor in a fourth case. These studies provide further evidence that some cases previously interpreted as malignant or regressing atypical histiocytosis and some types of HD are actually T cell disorders.
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STEIN ET AL have recently described a large-cell lymphoma of activated lymphoid cells expressing a Hodgkin's disease–associated antigen, Ki-1. This Ki-1 lymphoma is further distinguished histologically by a sinus pattern of lymph node infiltration simulating metastatic carcinoma or malignant histiocytosis. Among the 45 patients described by Stein et al the diagnostic biopsy site was the skin in three cases and lymph node in all but two of the remaining cases. Twenty percent of the patients were less than 20 years of age.

Our purpose is to describe a clinicopathologic syndrome of frequently regressing skin lesions and peripheral lymphadenopathy among children and adolescents with Ki-1 lymphoma. These cases often presented a diagnostic dilemma because of their unusual clinical manifestations and histology suggesting malignant or regressing atypical histiocytosis.[2,3] However, immunologic, cytochemical, and gene rearrangement studies revealed an activated T cell origin for the malignant cells in four of six cases.

MATERIALS AND METHODS

Three patients with similar but distinctive clinical features, histology, and tumor cell phenotype were identified initially among a series of 47 unselected patients from multiple institutions with childhood lymphomas, an incidence of 6%. Upon reporting these results,[4] three additional cases with similar features were then referred to one of us (M.E.K.), making a total of six patients presented in this study. All patients were under 20 years of age.

In five cases, both lymph node and skin biopsy results were available for study. In a sixth case, only a biopsy of skin was taken. All patients had examination of peripheral blood, bone marrow aspirate, and trephine biopsy for tumor cells. Lymph nodes were fixed in B5 for routine histology. Skin biopsies were fixed in either B5 or formalin, although B5 gave better morphology of lymphoid cells.

In each case, a corresponding piece of unfixed tissue was snap-frozen in isopentane or acetone cooled by dry ice and then used to prepare frozen sections of 5 to 6 mμ in thickness. Frozen sections were studied with monoclonal antibodies against cellular activation antigens including antigens expressed preferentially by Reed-Sternberg cells of Hodgkin's disease[5-7] (Ki-1, Leu-M1, HLA-DR, Tac, OKT9); T cell–specific antigens (T11,[8] Leu-1,[9] Leu-4,[10] T4,[11] T8,[12] 3A1[13]); B cell–specific antigens (HLB-3,[14] B1,[15] B4,[16] Leu-12,[17] Leu-14[18]) and surface immunoglobulin heavy and light chains (IgM, IgD, κ, λ); macrophage-specific antigens (OKM1,[19] Leu-M3,[20] Ki-M1,[21] Ki-M2,[21] Ki-M3,[21] Ki-M4[21]); and leukocyte common antigen (T200[22]) by use of an indirect immunoperoxidase method[23] or, in case 5, an alkaline phosphatase/antialkaline phosphatase technique.[24] A tumor cell suspension from case 1 was studied for rosette formation with sheep erythrocytes, IgG-coated bovine erythrocytes, and complement-IgM–coated erythrocytes as previously described.[25] Direct touch imprints or cytocentrifuge preparations of tumor cells were studied for the cytochemical enzymes myeloperoxidase, Sudan black B, acid phosphatase, and alphanaphthyl acetate or butyrate esterase as previously reported.[25]

DNA extractions were performed as described previously.[26] Briefly, high–molecular weight DNA was prepared from a tumor cell suspension from patient 1. Cells were incubated overnight at 37 °C in 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg/mL proteinase K. This was followed by phenol-chloroform extraction and ethanol precipitation. DNA was digested with appropriate restriction enzymes, size-fractionated by agarose gel electrophoresis, and transferred onto nitrocellulose paper by the method of Southern.[27] Filters were hybridized to nick-translated, 32p-labeled probes of the immunoglobulin heavy- and light-chain loci and of the β-chain of the T cell receptor. The filters were then washed at 55 °C in 0.1% SDS and 0.15 mol/L NaCl–0.0015 mol/L Na citrate prior to autoradiography.

RESULTS

Clinical findings. The clinical features at presentation as well as therapy, and follow-up data are summarized in

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Table 1. Presenting Clinical Features, Initial Pathologic Diagnosis, and Results of Treatment

	Case I	Case 2	Case 3	Case 4	Case 5	Case 6
Age/Sex/Race	13/M/Asian	8/M/white	12/M/white	12/F/black	19/F/white	11/M/black
Presenting chief complaint	tender lymph nodes and subcutaneous nodules of abdominal wall	skin nodule, right part of neck, "? insect bite or ringworm infection"	nodules on right part of neck and chest wall, "? insect bites"	enlarged lymph node "cat scratch"	antecubital mass ? abscess	ulcerating skin lesions on buttock, thigh, and arm
Initial pathologic diagnosis	malignant or regressing atypical histiocytosis	large-cell lymphoma or malignant histiocytosis	reactive or inflammatory *v* large-cell lymphoma	Hodgkin's disease *v* malignant histiocytosis	granulomatous inflammation, squamous carcinoma, regressing atypical histiocytosis	large-cell lymphoma *v* malignant histiocytosis
Constitutional symptoms	0	fever	0	fever	fever	0
Peripheral lymphadenopathy	I, C, A	C	C, A	E	A	0
Skin lesions	+	+	+	+	+	+
Therapy (duration)	local radiation (1,400 rad), CHOP (6 mo), COMP (12 mo)	COMP (6 mo) and local radiation (2,400 rad)	COMP, daunomycin IV + IT methotrexate (8 mo)	radiation to involved field, mantle, para-aortic lymph nodes, and splenic pedicle	local radiation (4,200–5,000 rad)	COMP + IT MTX, Ara-C, then modified LSA_2L_2 (6 mo)
Response to therapy	relapse of cutaneous nodules on left arm 4 mo after therapy. Added chemotherapy, whole-body radiation, and bone marrow transplant	complete remission	relapse of cutaneous nodules with hilar and retroperitoneal adenopathy, systemic symptoms 3 mo after therapy	relapse in skin of right breast	immediate relapse in abdomen treated with PROMACE-MOPP	complete remission
Survival (months from onset)	34	14	14	8	20	14
Survival (months from diagnosis)	30	14	13	6	4	13
Current status	no recurrence 7 mo post-BMT	alive without evidence of disease	alive	dead	alive	alive without evidence of disease

Abbreviations: I, inguinal; C, cervical; A, axillary; E, epitrochlear; BMT, bone marrow transplant; IV, intravenous; IT, intrathecal; CHOP, cyclophosphamide, adriamycin, vincristine, prednisone; COMP, cyclophosphamide, vincristine, methotrexate, prednisone; LSA_2L_2, cyclophosphamide, vincristine, prednisone, methotrexate, daunomycin, cytosine arabinoside, 6-thioguanine, L-asparginase, BCNU, hydroxyurea; PROMACE-MOPP, prednisone, methotrexate, adriamycin, cyclophosphamide, VP-16–nitrogen mustard, vincristine, procarbazine, prednisone.

Table 1. Four patients were male, and two were female. Their ages ranged from 8 to 19 years. Three were white, two black, and one of Asian ancestry. Four patients presented with painful nodular skin lesions around the neck, chest, and upper or lower extremities, suggesting clinical diagnoses of insect bite or abscess. Cutaneous nodules developed in the remaining patients soon after their initial presentation of localized, tender lymphadenopathy. In three cases the skin lesions underwent temporary spontaneous regression a few weeks after their initial appearance but reappeared at the same sites shortly thereafter. Three patients received a trial of antibiotics with no response. Three patients had fever, and a fourth patient had night sweats at presentation. In patient 3, who had no systemic symptoms at presentation, high fevers and weight loss developed upon relapse of his disease.

At presentation no patient had a mediastinal mass, hepatosplenomegaly, or symptoms of gastrointestinal obstruction. The bone marrow was not involved, and no abnormal cells were found in the blood. Hemoglobin, WBC count, and γ-globulin levels were within normal limits in all but the sixth patient who had a hemoglobin concentration of 10.2 g and a hematocrit value of 32%. Initial differential diagnoses of pathologists included malignant histiocytosis (four cases), regressing atypical histiocytosis (two cases), Hodgkin's disease (one case), large-cell (immunoblastic) lymphoma (three cases), squamous carcinoma (one case), or nonmalignant inflammatory disease (two cases). In four of the six cases, treatment was delayed for weeks to months while uncertainty about the initial pathologic diagnosis was resolved. Four patients experienced relapse of their disease shortly after completing therapy. Relapses were noted first in the skin in new and original sites and later in the chest and/or abdomen. Temporary regression of skin lesions in patient 3 and confusion of a large ulcerated skin lesion with radiation necrosis in patient 4 caused delay in recognizing recurrent disease.

Pathology. Lymph nodes showed similar architectural alterations in all cases. Sheets of basophilic or pale-staining tumor cells infiltrated the thymic-dependent paracortical regions and surrounded residual follicles of B lymphocytes (Fig 1). Moderate numbers of plasma cells and some small lymphocytes but no eosinophils or neutrophils were found among the sheets of tumor cells. Occasional diffuse fibrosis but no broad sclerotic bands of collagen were present. Most distinctive was the focal, often massive infiltration of subcapsular sinuses imparting an appearance of metastatic tumor or malignant histiocytosis (Fig 2). A diagnosis of histiocytosis was supported by tumor cell erythrophagocytosis in touch imprints of a lymph node from case 1. Multinucleate Reed-Sternberg or Reed-Sternberg–like cells were sometimes found in a cytoarchitectural milieu that was consistent with Hodgkin's disease (Fig 3).

In skin, there was no primary infiltration of the epidermis (epidermotropism) and no cells with cerebriform nuclei, making a distinction from mycosis fungoides possible in all cases. The tumor cell infiltrate was confined to the lower reticular dermis in four cases, but in two cases (nos. 4 and 6), the infiltrate was more extensive and involved the entire dermis, destroying skin appendages and focally ulcerating the epidermis (Fig 4). As shown in this Figure, the tumor cells in case 6 consisted of highly atypical mononuclear and multinucleated cells. Bone marrow biopsy specimens were normocellular in all but case 4 in which the marrow was slightly hypercellular. None of the specimens contained fibrosis or evidence of tumor cell infiltration.

Cytochemistry. Four cases were studied cytochemically. In no case was there staining of tumor cells for myeloperoxi-

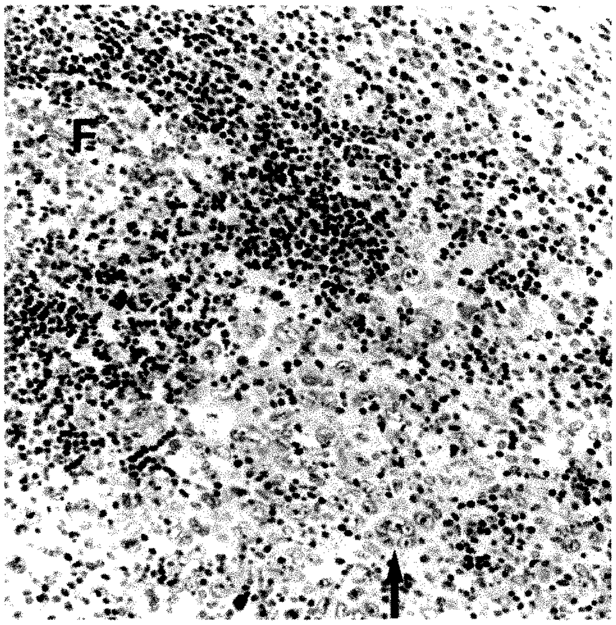

Fig 1. Large pale-staining tumor cells, including multinucleated cell (arrow) surround residual follicle (F) in lymph node of patient 5 (Hematoxylin-eosin stain [HE]: original magnification × 200).

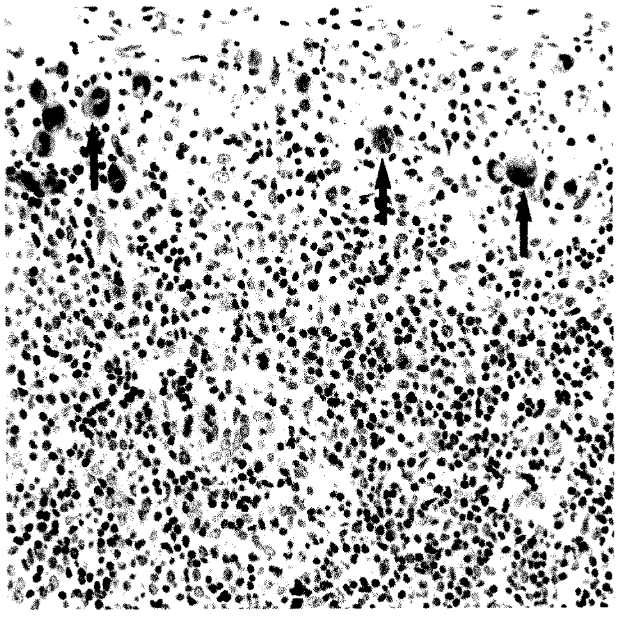

Fig 2. Subcapsular sinus infiltration of multinucleate tumor cells (arrows) in lymph node of patient 5 (HE; original magnification × 200).

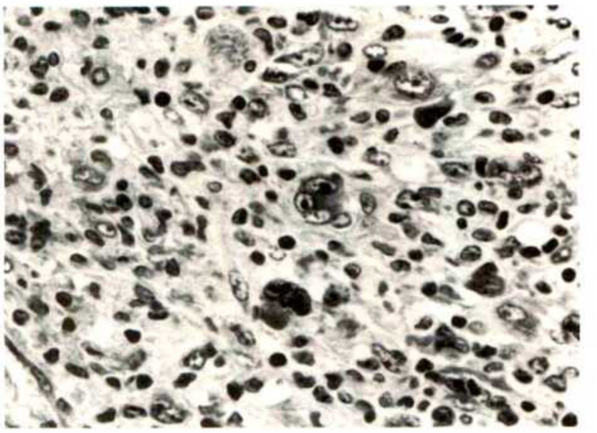

Fig 3. Reed-Sternberg or Reed-Sternberg–like cell in cellular environment consistent with Hodgkin's disease from lymph node of patient 5 (HE; original magnification × 400; current magnification × 300).

dase-, Sudan black B–, or periodic acid–Schiff–positive material. In case 1, most tumor cells contained a slight to moderate number of acid phosphatase–positive granules. In no case did tumor cells show diffuse staining for nonspecific esterases (macrophage pattern), but in case 3, 10% of cells stained focally in a pattern similar to that of mature T lymphocytes.[28]

Immunohistochemistry. Interpretation of immunoperoxidase stains was facilitated by the focal or compartmental tumor cell infiltration of lymph nodes and dense tumor cell infiltration of skin. Tumor cells from cases 1 through 5 were nonreactive with a panel of monoclonal antibodies directed against surface determinants of macrophage/histiocytes (Table 2). No surface or cytoplasmic immunoglobulin or surface B cell antigens were found. In cases 5 and 6 some or all of the large tumor cells expressed the T cell antigen T11. Moreover, in case 5, there was staining of some tumor cells for the helper T cell antigen T4 although the exact percentage of cells stained was difficult to determine because of some background staining of tissue macrophage/histiocytes. In all cases the large majority of tumor cells expressed the cellular activation antigens HLA-DR (Ia-like), TAC and T9, and the Hodgkin's disease–associated antigen Ki-1 (Fig 5). However, in contrast to most types of Hodgkin's disease other than the lymphocyte predominance type,[29] staining of tumor cells for Leu-M1 antigen was negative (weak or equivocal Leu-M1 staining was found in cytocentrifuge preparations of case 1). Also, unlike most forms of Hodgkin's disease other than the lymphocyte predominance type,[30,31] staining of tumor cells for common leukocyte antigen T200 was found in all cases.

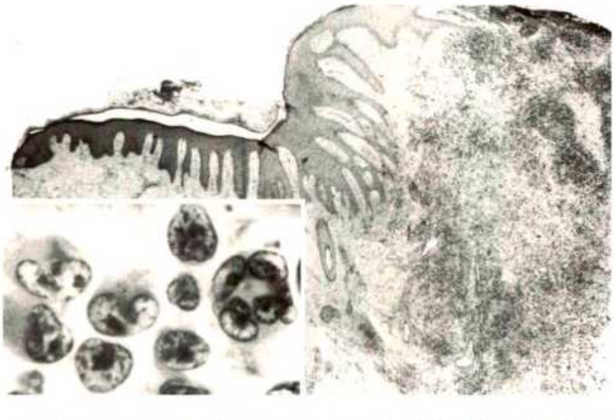

Fig 4. Lymphoid infiltration in ulcerated skin nodule of patient 6 (HE; original magnification × 30; current magnification ×22); inset of pleomorphic tumor cells, original magnification × 1,000; current magnification × 740).

Rosette studies. In contrast to small normal T cells, the tumor cells in case 1 did not form E rosettes with sheep erythrocytes. Also, in contrast to normal macrophage-histiocytes, the tumor cells in this case did not demonstrate surface receptors for IgG-coated erythrocytes or IgM- and complement-coated erythrocytes and showed no in vitro phagocytosis of these reagents.

Detection of T cell receptor β-chain rearrangement. We detected a unique rearrangement of the constant region Cβ2 in DNA from patient 1. As demonstrated in Fig 6, digestion with *Eco*RI and hybridization to the T cell receptor β-chain probe revealed only germline fragments. Hybridization of the probe to *Xba*I digests of the same DNA, however, revealed a third band in addition to the two predicted germline bands. This is consistent with the presence of a monoclonal proliferation of T cells with a unique rearrangement of Cβ2.[32] Similar studies using probes of the immunoglobulin gene loci showed only germline configuration of the DNA, consistent with a non–B cell origin of the tumor cells.

DISCUSSION

The six cases of childhood lymphoma reported here are remarkable for their similar clinical features, histopathology, and immunophenotype of tumor cells. Each patient had rapidly growing skin lesions. Signs of inflammation and temporary regression of skin lesions in three cases suggested a nonneoplastic process, and in two cases a reaction to an insect bite was suspected. Lymphadenopathy was confined initially to peripheral, usually regional, lymph nodes. The usual sites of disease in other childhood lymphomas were not initially affected. At presentation, there was no mediastinal or intra-abdominal mass and no bone marrow or CNS involvement. However, mediastinal and intra-abdominal disease eventually developed in two of our patients and was found at presentation in some other children with Ki-1 lymphoma who had no skin lesions and are not included in this report.

The pathologic diagnosis was difficult in these cases. Because of the unusual histology and the unfamiliarity of most pathologists with this clinicopathologic syndrome, a variety of other diagnoses was reasonably made, including malignant or regressing histiocytosis, squamous carcinoma, Hodgkin's disease, or inflammation. A similar experience has been recorded for adult lymphomas of this histology and immunophenotype.[1] The distinctive pattern of lymph node sinus and paracortical infiltration by large pleomorphic cells was consistent with malignant histiocytosis or metastatic carcinoma. The presence of Reed-Sternberg or Reed-Stern-

Table 2. Immunologic Results

Markers	Antibody	Patient					
		1	2	3	4	5	6
Hodgkin's disease associated	Ki-1	+	+	+	+	+	+
	Leu-M1	–	–	–	–	–	–
Activation antigens	HLA-DR	+	+	+	+	+	+
	TAC	+	+	+	+	+	+
	OKT9	+	+	+	+	+	+
T cell specific	T11	–	–	–	–	+	+
	Leu-1	–	–	–	–	–	–
	Leu-4	–	–	–	–	–	–
	T4	–	–	–	–	+	–
	T8	–	–	–	–	–	–
	3A1	–	–	–	–	NA	–
B cell specific	HLB-3	–	–	–	–	NA	NA
	B-1	NA	–	–	NA	NA	–
	B-4	NA	NA	–	NA	–	NA
	Leu-12	NA	NA	NA	–	NA	–
	Leu-14	–	NA	–	–	NA	NA
	sIg (D,M,K,L)	–	–	–	NA	–	–
Macrophage/histiocyte specific	OKM1	NA	NA	NA	–	–	NA
	Leu-M3	NA	NA	NA	–	–	NA
	Ki-M1	–	–	–	–	NA	NA
	Ki-M2	–	–	–	–	NA	NA
	Ki-M3	–	–	–	–	NA	NA
	Ki-M4	–	–	–	–	NA	NA
Hematopoietic	T-200	+	+	+	+	+	+

Abbreviation: NA, not assessed.

berg–like cells was suggestive of Hodgkin's disease. However, the large sheets of tumor cells, lack of reactive cells, and extensive sinus involvement would be unusual for Hodgkin's disease. Ulceration of skin lesions with associated inflammation and temporary regression of skin lesions caused some hesitation about a diagnosis of malignancy.

Immunophenotyping of tumor cells helped to clarify the diagnosis and establish the distinctive character of this disorder. In each case the majority of tumor cells expressed the Hodgkin's disease–associated antigen Ki-1, but unlike most types of Hodgkin's disease,[29] the Reed-Sternberg–like cells in these cases were not reactive with antibody Leu-M1. A further distinction from Hodgkin's disease was the finding that the tumor cells were positive for common leukocyte antigen T200, an antigen usually not expressed on Reed-Sternberg cells.[30,32] This also helped to distinguish these cases from metastatic carcinoma or sarcoma, which are negative for common leukocyte antigen.[33] An absence of macrophage-histiocyte antigens, lack of tumor cell rosette formation or phagocytosis of IgG-coated erythrocytes, and lack of diffuse cytoplasmic staining for nonspecific esterase distinguished these cases from malignant or regressing atypical histiocytosis.

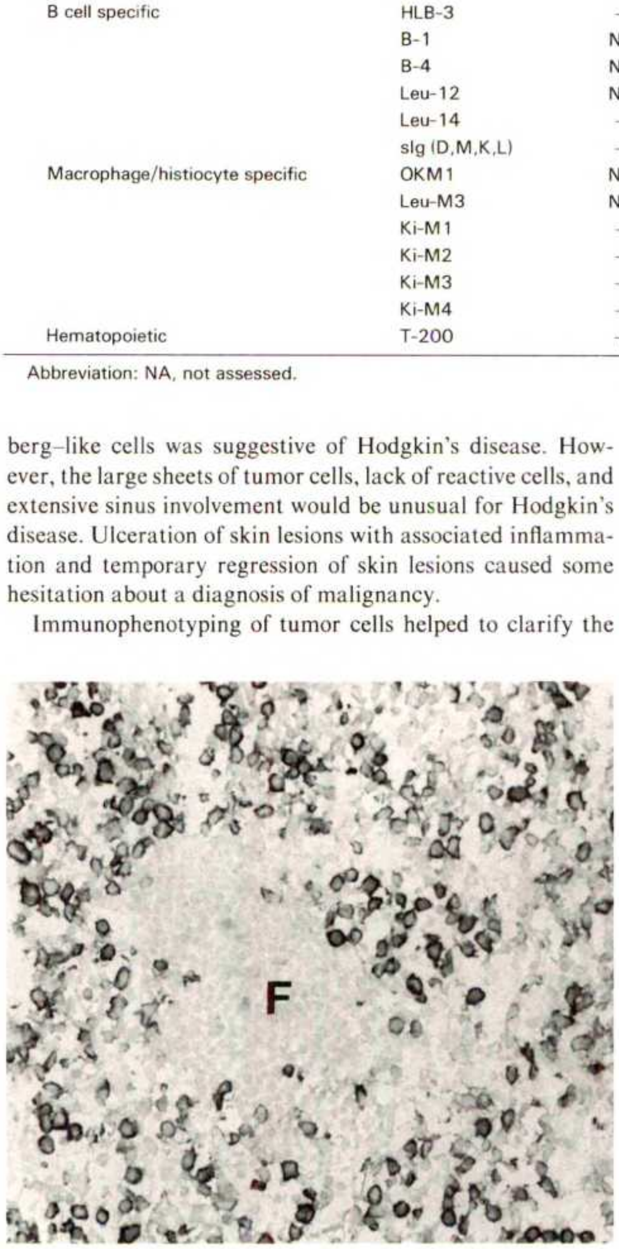

Fig 5. Ki-1–positive tumor cells surround unstained lymph node follicle (F) in patient 3 (immunoperoxidase stain of frozen section; original magnification × 200).

The T cell nature of the malignant cells was established by DNA analysis and studies with T cell–specific monoclonal antibodies in three cases. The pattern of cytochemical staining for nonspecific esterase and tumor cell infiltration of thymic-dependent regions of lymph nodes was consistent with a T cell origin for the tumor cells in a fourth case. Similar cytochemical staining can sometimes be observed in certain B cell tumors and interdigitating reticulum cells.[6] However, the tumor cells lacked several B cell antigens, surface and cytoplasmic immunoglobulin, and KiM1, KiM2 antigens, which mark interdigitating reticulum cells. Thus the balance of evidence seems to favor a T cell origin for the majority of our cases as it does in the majority of Ki-1 lymphomas of adults.[1]

In one case (no. 5), the phenotype of tumor cells was

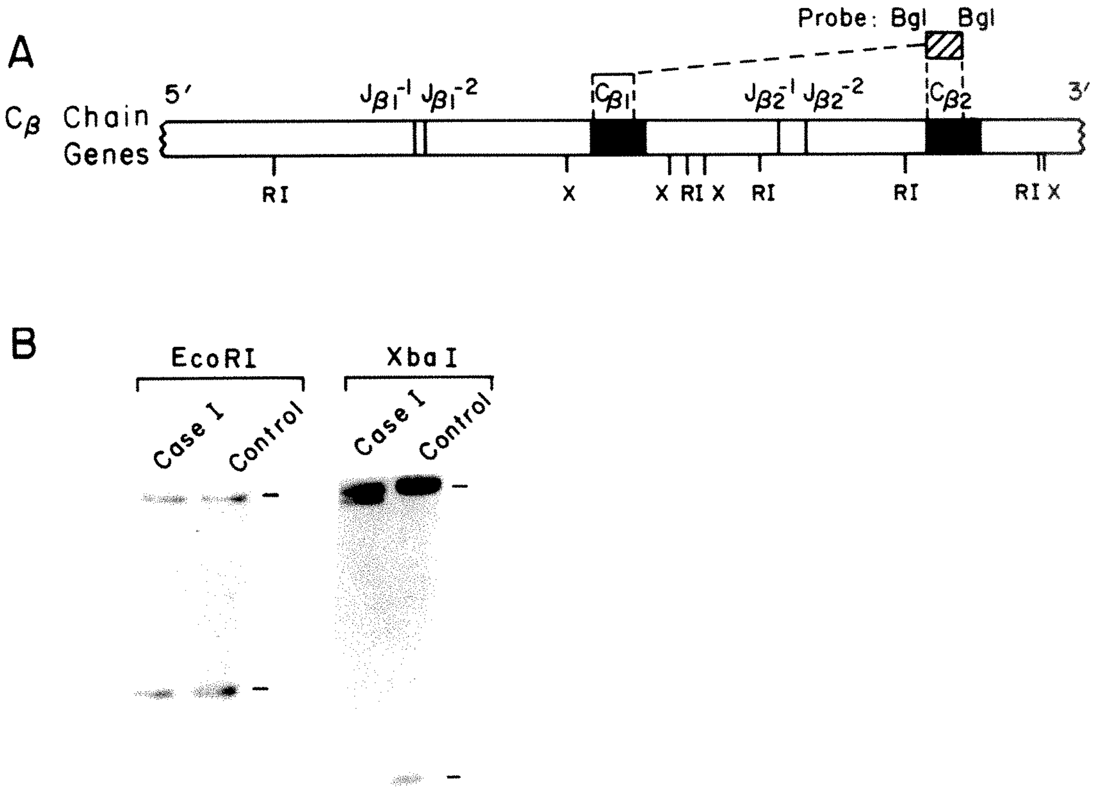

Fig 6. Organization of the human T cell receptor β chain genes is shown in (A). The two constant region genes ($C\beta 1$ and $C\beta 2$) are diagrammed. Southern blot analysis was performed using a *BglII*- *BglII* probe from $C\beta 2$ that hybridizes equally to both constant regions. DNA was digested with *EcoRi* or *XbaI*. The restriction sites for those enzymes are shown. As illustrated in (B), digestion with *EcoRI* revealed two hybridizing fragments consistent with a germline configuration of the $C\beta 1$ genes. Digestion with *XbaI* and hybrization to the probe revealed a third band that was not seen on digests of control DNA, consistent with a monoclonal population of cells with a unique rearrangement of the $c\beta 2$ genes. The germline fragments are marked by dashes.

further defined as that of an activated helper T cell expressing Ia, Tac, T9, T11, and T4 antigens. The phenotype of activated helper T cells expressing Hodgkins's disease–associated antigens has been reported previously by one of us (M.E.K.) in lymphomatoid papulosis, a chronic, usually self-healing skin eruption seen mainly in adults.[34] However, in contrast to the patients described here who were clinically ill and had rapid development of lymphadenopathy in five of six cases, extracutaneous disease develops slowly in only a small proportion (4% to 20%) of patients with lymphomatoid papulosis.[35] A helper T cell lymphoma involving lymph nodes and skin in five adults was recently reported by van den Oord et al.[36] However, they did not describe the peculiar pattern of lymph node involvement or the unique Ia+, Ki-1+ phenotype of tumor cells observed in our cases. Nevertheless, the skin lesions of their patients were also nodular and non-epidermotropic. Grogan et al also reported nodular skin lesions in six of 11 elderly patients with aggressive peripheral T cell lymphomas.[37] Therefore, our patients may be part of a spectrum of patients of all ages who have a similar disorder.

Stein et al have recently proposed a lymphoid origin for the tumor cells of Hodgkin's disease and certain large-cell lymphomas that resemble malignant histiocytosis.[1] Several of our Ki-1–positive large-cell lymphomas were initially interpreted as malignant histiocytosis or Hodgkin's disease, in part because no T or B cell antigens were detected. However, in one of these cases it was possible to demonstrate an underlying rearrangement of the genes for the β-chain of the T cell receptor. This is evidence supporting the concept that some types of Hodgkin's disease and other large-cell lymphomas that express Ki-1 antigen but lack lymphoid surface antigens may in fact be of T cell origin.

It is therefore possible that some cases previously reported as cutaneous malignant or regressing atypical histiocytosis (RAH)[2,3] are actually T cell disorders. Our case 1 in which there was temporary regression of skin lesions and tumor cell erythrophagocytosis, both characteristics of RAH,[2] had no lymphoid surface antigens but was proven to be a T cell disorder by gene rearrangement studies (Fig 6). In our case 6 there were other features of RAH[2] such as epitheliomatous epidermal hyperplasia, frequent mitoses, and highly atypical mononuclear and multinucleated cells (Fig 4). The tumor cells of our case 5 had a similar phenotype (expressing OKT11, but negative for Leu-4 (T3), Leu-1, and T4) to that of tumor cells in a case recently reported as cutaneous malignant histiocytosis by Dodd et al.[3]

Thus it seems that these T cell lymphomas of the skin may be related to other extranodal T cell malignancies such as those involving the intestine in patients with celiac sprue that originally were thought to be malignant histiocytosis.[38,39]

Erythrophagocytic T γ-lymphoma, which initially involves the spleen, is another T cell malignancy resembling malignant histiocytosis.[25] Similar to the present cases, this T cell lymphoma showed a distinctive infiltration of sinuses and paracortical thymic-dependent regions of lymph nodes. Further immunologic and gene rearrangement studies can be expected to better define the broad spectrum of pathology and clinical behavior of T cell malignancies. Although the natural history and optimal therapy for this type of lymphoma (Ki-1 lymphoma) have not been defined, several points may be learned from this group of patients. The disease may present with spontaneously regressing skin lesions, causing confusion about a diagnosis of malignancy. Two patients who received prompt administration of systemic combined drug chemotherapy have had complete remissions sustained for more than 1 year, whereas four patients who received radiation without chemotherapy or had delayed administration of chemotherapy had relapses in less than 6 months. Improved results are anticipated as clinicians and pathologists become more familiar with this disorder, leading to earlier diagnosis and therapy.

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REFERENCES

1. Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, Gattler K, Falini B, Delsol G, Lemke H, Schwarting R, Lennert K: The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic tissue. Evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood 66:848, 1985

2. Dodd HJ, Stansfeld AG, Chambers TJ: Cutaneous malignant histiocytosis—A clinicopathological review of five cases. Br J Dermatol 113:455, 1985

3. Flynn KJ, Dehner LP, Gajl-Peczalska KJ, Dahl MV, Ramsay N, Wang N: Regressing atypical histiocytosis: A cutaneous proliferation of atypical neoplastic histiocytes with unexpectedly indolent biologic behavior. Cancer 49:959, 1982

4. Kadin ME, Schweid A, Ireland K, Dorfman RF: Ki-1 lymphoma in childhood. Lab Invest 52:32, 1985

5. Schwab V, Stein H, Gerdes J, Lemke H, Kirchner H, Schaadt M, Diehl V: Production of a monoclonal antibody specific for Hodgkin and Sternberg-Reed cells of Hodgkin's disease and a subset normal lymphoid cells. Nature 299:65, 1982

6. Kadin ME: Possible origin of the Reed-Sternberg cell from an interdigitating reticulum cell. Cancer Treat Rep 601:608, 1982

7. Hsu SM, Yang K, Jaffe ES: Phenotypic expression of Hodgkin's and Reed-Sternberg cells in Hodgkin's disease. Am J Pathol 118:209, 1985

8. Kamoun M, Martin PJ, Hansen JA, Brown MA, Siadak AW, Nowinski RC: Identification of a human T lymphocyte surface protein associated with the E-rosette receptor. J Exp Med 153:207, 1981

9. Martin PJ, Hansen JA, Nowinski TC, Brown MA: A new human T cell differentiation antigen: Unexpected expression on chronic lymphocytic leukemia cells. Immunogenetics 11:429, 1980

10. Reinherz EL, Kung PC, Goldstein G, Schlossman SF: A monoclonal antibody with selective reactivity with functionally mature human thymocytes and all peripheral mature T cells. J Immunol 123:1312, 1979

11. Ledbetter JA, Evans RL, Lipinski M, Cunningham-Rundles C, Good RA, Herzenberg LA: Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and man. J Exp Med 153:310, 1981

12. Evans RL, Wall DW, Platsoucas CD, Siegal FP, Fikrig SM, Testa CM, Good RA: Thymus-dependent membrane antigens in man: Inhibition of cell-mediated lympholysis by monoclonal antibodies to the TH2 antigen. Proc Natl Acad Sci USA 78:544, 1981

13. Haynes BF, Eisenbarth GS, Fauci AS: Human lymphocyte antigens: Production of a monoclonal antibody that defines functional thymus-derived lymphocyte subsets. Proc Natl Acad Sci USA 76:5829, 1979

14. Kikuchi T, Koshiba H, Kasai K, Hiratsuka H, Fujimoto N, Vede T: B-cell malignancies and differentiation antigens defined with monoclonal antibodies. Jpn J Clin Oncol 13:517, 1983

15. Stashenko P, Nadler LM, Hardy R, Schlossman SF: Characterization of a human B cell–specific antigen. J Immunol 125:1678, 1980

16. Nadler LM, Anderson KC, Marti G, Bates M, Park E, Daley JF, Schlossman SF: B4, a human B lymphocyte associated antigen expressed on normal, mitogen activated and malignant B lymphocytes. J Immunol 131:244, 1983

17. Meeker TC, Miller R, Link M, Bindl J, Warnke R, Levy R: A unique human B lymphocyte antigen defined by a monoclonal antibody. Hybridoma 3:305, 1984

18. Schwarting R, Stein H, Wang CY: Monoclonal antibodies S-HCL1 and S-HCL3 allow the diagnosis of hairy cell leukemia. Blood 65:974, 1985

19. Arnaout MA, Todd RF, Dana N, Melamed J, Schlossman SF, Colten HR: Inhibition of phagocytosis of complement C3' or immunoglobulin G–coated particles and of C3bi binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein (Mo1). J Clin Invest 72:171, 1983

20. Dimitriu-Bona A, Burmester GR, Waters SJ, Winchester RJ: Human mononuclear phagocyte differentiation antigens. I. Patterns of antigen expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J Immunol 130:145, 1983

21. Radzun HJ, Parwaresch MR: Differential immunohistochemical resolution of the human mononuclear phagocyte system. Cell Immunol 82:174, 1983

22. Omary MB, Trowbridge IS, Battifora HA: Human homologue of murine T200 glycoprotein. J Exp Med 152:842, 1980

23. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577, 1981

24. Cordell JL, Falini B, Erber W, Ghoshak, Abdulozzi A, MacDonald S, Pulford KAF, Stein H, Mason DY: Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal alkaline phosphatase (APAAP) complexes. J Histochem Cytochem 32:219, 1984

25. Kadin ME, Kamoun M, Lamberg J: Erthrophagocytic T gamma lymphoma. A clinicopathologic entity resembling malignant histiocytosis. N Engl J Med 304:648, 1981

26. Bell GI, Karam JH, Rutter WJ: Polymorphic DNA region adjacent to the 5' end of the human insulin gene. Proc Natl Acad Sci USA 78:5750, 1981

27. Southern EM: Detection of specific sequences among DNA

fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

28. Pinkus GS, Hargreaves HK, McLeod JA, Nadler LM, Rosenthal DS, Said JW: Naphthyl acetate esterase activity—A cytochemical marker for T lymphocytes. Correlation with immunologic studies of normal tissues, lymphocytic leukemias, non-Hodgkin's lymphomas, Hodgkin's disease and other lymphoproliferative disorders. Am J Pathol 97:17, 1979

29. Pinkus GS, Premi T, Said JW: Leu M1—A marker for Reed-Sternberg cells in Hodgkin's disease. An immunoperoxidase study of paraffin-embedded tissues. Am J Pathol 119:244, 1985

30. Kurtin PJ, Pinkus GS: Leukocyte common antigen—A diagnostic discriminant between hematopoietic and nonhematopoietic neoplasms in paraffin sections using monoclonal antibodies: Correlation with immunologic studies and ultrastructural localization. Hum Pathol 16:353, 1985

31. Pinkus GS, Said JW: Hodgkin's disease, lymphocyte predominant type, nodular—A distinct entity? Unique staining profile for L&H variants of Reed-Sternberg cells defined by monoclonal antibodies to leukocyte common antigen, granulocyte specific antigen and B-cell specific antigen. Am J Pathol 118:1, 1985

32. Duby AD, Klein KA, Murre C, Seidman JG: A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 228:1204, 1985

33. Battifora H, Trowbridge IS: A monoclonal antibody useful for the differential diagnosis between malignant lymphoma and nonhematopoietic neoplasms. Cancer 51:816, 1983

34. Kadin ME, Nasu K, Sako D, Said J, Vonderheid E: Lymphomatoid papulosis: A cutaneous proliferation of activated helper T cells expressing Hodgkin's disease associated antigens. Am J Pathol 119:315, 1985

35. Sanchez NP, Pittelkow MR, Muller SA, Banks PM, Winkelman RK: The clinicopathologic spectrum of lymphomatoid papulosis: Study of 31 cases. J Am Acad Dermatol 8:81, 1983

36. Van den Oord JJ, DeWolf-Peeters C, Frizzera G, Desmet VJ, Tricot G, Thomas J, Marien K, Gatter KC, Mason DY: T-helper cell lymphoma involving lymph nodes and skin. A clinical, morphological and immunohistochemical analysis of five cases. Cancer 55:1714, 1985

37. Grogan TM, Fielder K, Rangel C, Jolley CS, Wirt DP, Hicks MJ, Miller TP, Brooks R, Greenberg B, Jones S: Peripheral T-cell lymphoma: Aggressive disease with heterogeneous immunotypes. Am J Clin Pathol 83:249, 1985

38. Isaacson PG, Spencer J, Connolly CE, Pollock DJ, Stein H, O'Connor NTJ, Bevan DH, Kirkham N, Wainscoat JS, Mason DY: Malignant histiocytosis of the intestine: A T-cell lymphoma. Lancet:688, 1985

39. Loughran TP, Kadin ME, Deeg HJ: T-cell intestinal lymphoma associated with celiac sprue. Ann Intern Med 104:44, 1986

Erythrocyte–Endothelial Cell Adherence in Sickle Cell Disorders

By Brian D. Smith and Paul L. La Celle

Detachment of individual sickle erythrocytes from cultured endothelial cell monolayers has been evaluated by a fluid-shearing technique in an effort to quantitate adherence at shear forces that would be anticipated in the in vivo circulation. Nonirreversibly sickled cells (non-ISC) were more adherent at normal oxygen tensions than control cells. More than 1% non-ISC remained attached to the monolayer at forces greater than physiologic shear stresses in capillary and venous circulations, and many of the most avidly attached cells, once separated, immediately reattached to adjacent endothelial cells. These data suggest that hemoglobin S-containing erythrocytes may have a higher frequency of adherene in vivo in regions of low shear stress where prolonged erythrocyte–endothelial cell contact could occur. Some of these cells detached by shear force would subsequently reattach in in vivo conditions. Plasma-enhanced attachment frequency and plasma from blood in a case of sickle crisis caused further increase. These observations further support the concept that sickle erythrocyte–endothelial cell interaction may be a significant factor in initiation of vascular occlusive events in sickle cell disease.

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AN INCREASED FREQUENCY of erythrocyte adherence to the vascular endothelium has been postulated as a contributing factor in the vascular obstructive phenomenon of sickle cell anemia.[1,2] In this disorder the flow disturbances range from microvascular occlusive crises to arterial thromboses; these may result from both intrinsic erythrocyte abnormalities and plasma changes.[3] The well-known rheologic abnormalities of the sickle cell are manifested in vitro by the elevated viscosity of packed cell suspensions[3] and decreased filterability, and in vascular occlusive crises with attendant elevation of levels of reactive proteins the abnormalities are greater.[4,5] In small in vitro channels of the capillary size range, there is an observed decrease in the hematocrit value[6] with reduction of apparent viscosity, findings also noted in normal animal models.[7] The relatively low hematocrit value in sickle cell disease may result in a lower than normal capillary hematocrit value that would compensate for the increased viscosity and related increase in vessel wall shear stress that occur with the loss of cellular deformability[8] demonstrable in the irreversibly sickled cell (ISC) portion of sickle erythrocytes.[9] A decrease in wall shear stress particularly in vessels typified by lower flow velocity would tend toward increased blood cell–endothelial cell contact and favor adherence, and if the sickle cell surface characteristically were more adhesive, these flow dynamics and the sickle cell tendency for adherence could have important implications for the pathophysiology of the disorder. Adherence of erythrocytes to the endothelium would be expected to contribute to abnormal flow dynamics by changing the local vessel geometry and thereby favor further cell accumulation and resultant occlusion. The specific mechanism by which adherence of sickle cells to the endothelium may produce its effect in occlusion has not been demonstrated; however, in vitro observations indicate a correlation between increased adherence observed in vitro and clinical severity,[10] and during the crisis state, the rate of erythrocyte–endothelial cell adherence increases.[11,12]

The postulated increased frequency of an in vivo adherence to endothelium has been interpreted from in vitro observations of sickle erythrocytes binding to cultured endothelial cells. In these experiments, binding was assessed by sequential washing of isotope-labeled erythrocytes from endothelial monolayer cultures with the assumption that residual binding after washes reflects the likelihood of adherence in vivo. The studies of Hebbel et al showed that 0.1% to 1.0% of sickle cells remained adherent, thus demonstrating unique characteristics of sickle cell–endothelial cell interaction and strongly supporting the proposed mechanism.[10–12]

More recently, Mohandas and Evans[13] have developed a direct quantitive measure to examine sickle-endothelial cell adherence with results that demonstrated that 90% of sickle cells adhered in the presence of autologous plasma and one third of normal cells adhered in the presence of sickle plasma. The strength of sickle cell adhesion was nearly 50% greater than the control. Burns et al[14] did not detect significant differences between adherence properties of sickle cells and normal control cells in systems providing laminar flow conditions, but with vortex flow that promotes erythrocyte–endothelial cell contact, sickle cells were significantly more adherent than control cells.

The purpose of the present study was quantitation of the retention of adherent sickle erythrocytes by endothelial cells as a function of applied fluid shear stress to determine whether erythrocytes remain attached at stresses typical of in vivo rheologic conditions. An abnormal frequency of adherence was observed at stresses typical of the calculated physiologic shear stresses of the microcirculation and arteries, thus providing support for the concept of a specific role for erythrocyte–endothelial cell interaction in the pathophysiology of sickle cell disease.

MATERIALS AND METHODS

Patients. Blood samples from five patients with sickle cell anemia confirmed by electrophoresis were studied. Two patients

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(I.D. and A.H.) had no history of vascular crisis, one (T.J.) had been admitted for treatment of vaso-occlusive crisis, and two (A.T. and T.W.) had previous experience of vascular crisis but were asymptomatic at the time of study. Two patients (D.H. and R.M.) had sickle-thalassemia, were asymptomatic, and had no history of vascular crisis. Tables 1 and 2 indicate the study group. Blood samples from 12 healthy volunteers served as controls. Informed consent was obtained in each instance.

Erythrocytes. Venous blood was collected with citrate or heparin as anticoagulant. There was no differnce in results obtained with one anticoagulant compared with the other, and no coagulation was apparent during the incubation experiments. Samples were centrifuged at 2,300 g for 15 minutes to separate cells and plasma, and the plasma was centrifuged subsequently at 6,500 g for 30 minutes to remove platelets. Some erythrocytes were suspended in autologous plasma and others washed three times in isotonic phosphate buffer at physiologic pH and resuspended in this buffer. Suspensions for layering on the endothelial monolayer, glass, or plastic surfaces contained washed or unwashed cells mixed with either phosphate buffer plus albumin (5 g/L) or autologous plasma. To aid visualization final hematocrit values were less than 1%; however, there was no change in adherence, with hematocrit values ranging from less than 1% to 25%.

Endothelial cell cultures. Human umbilical veins were cannulated and infused with phosphate buffer to remove erythrocytes, then filled with Pronase (Calbiochem-Behring Corp, San Diego) for 20 minutes at 37 °C to loosen endothelial cells. The endothelial cells were collected, washed twice in hypotonic buffer to lyse contaminating erythrocytes, mixed with McCoy's M-199 or 5A/1X powder culture media (GIBCO, Grand Island, NY), and layered on multiple 1-cm squares of plastic or glass. Thus, endothelial cells from the same cord were used for both control and sickle cell studies, which gave consistent, reproducible results. The culture medium was changed under sterile conditions at 24- to 48-hour intervals until the endothelium formed a confluent monolayer.

Quantitation of erythrocyte adherence. Erythrocytes in phosphate-albumin buffer were allowed to settle for 30 minutes at 37 °C, forming a layer two to five cells deep on an endothelial monolayer. Under direct microscopic visualization and closed circuit video recording for later analysis, adhesion was studied on ten fields, each with an endothelial surface area sufficient to provide contact for 100 erythrocytes. An initial local wall shear stress of 1 dyne/cm^2, an order of magnitude less than that normally found in the microcirculation, was induced by flow from a 12-μm (internal diameter) glass pipette filled with phosphate buffer, causing nonadherent cells to move out of the field. Remaining adherent cells were subjected to progressively higher shear stress until separation occurred (Fig 1).

In the studies the pipette was placed 10 μm from the adherent erythrocyte, and positive pressure was applied to the pipette to produce flow that impinged on the cell. The 10-μm distance was measured on the video screen (see Fig 1). Calculation of shear stress on the erythrocytes resting on the endothelial surface was based on the assumption that the parabolic distribution of the velocity profile in the pipette is maintained approximately over the short interval between the pipette tip and the individual erythrocyte under study. The flow velocity, a function of applied pressure, was determined by observation of the velocity of small axially flowing particles in the buffer in the pipette. Since the pipette axis is nearly parallel to the endothelial surface, the actual force on the cell is approximately equal to the product of the wall shear stress and the projected area of the erythrocyte. However, since the projected area or cross section of the cell perpendicular to the direction of flow is fairly uniform because of the similarity of erythrocyte diameter and volume, the wall shear stress proportional to the force is a practical expression of the force on the cell and permits estimation of in vivo dynamics from the in vitro experiment.

Wall shear stress calculations for in vivo model pressure and vessel dimension measurements[15] provided approximate ranges for arterioles, capillaries, and venules. Calculations from other animal models demonstrated similar or lower values for in vivo wall shear stress.[16]

RESULTS

The fluid shear stress required for separation of adherent sickle erythrocytes from the endothelial cell monolayer is

Table 1. Erythrocytes Suspended in Albumin Plus Isotonic Buffer. Erythrocyte Adherence to Cultured Endothelium for Individual Patients

	Percent Adherence at Various Shear Stresses (SE)				
Subjects	1 dyne/cm^2	10 dyne/cm^2	20 dyne/cm^2	30 dyne/cm^2	55 dyne/cm^2
Sickle cell patients					
I.D.	3.30 (0.73)	2.80 (0.63)	2.80 (0.63)	2.70 (0.65)	2.30 (0.59)
A.T.	1.38 (0.13)	0.44 (0.05)	0.25 (0.03)	0.22 (0.03)	0.09 (0.01)
E.W.	2.00 (0.99)	2.00 (0.99)	2.00 (0.99)	2.00 (0.99)	2.00 (0.99)
T.J.	0.10 (0.10)	0.10 (0.10)	0.10 (0.10)	0.10 (0.10)	0.10 (0.10)
A.H.	0.82 (0.24)	0.41 (0.12)	0.30 (0.09)	0.23 (0.07)	0.06 (0.02)
Sickle-thalassemia patients					
P.H.	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.00
R.M.	0.43 (0.06)	0.29 (0.04)	0.29 (0.04)	0.14 (0.02)	0.07 (0.03)
Controls					
B.S.	0.60 (0.10)	0.21 (0.02)	0.15 (0.02)	0.10 (0.02)	0.08 (0.01)
C.S.	0.12 (0.04)	0.00	0.00	0.00	0.00
B.F.	0.00	0.00	0.00	0.00	0.00
D.K.	0.00	0.00	0.00	0.00	0.00
A.M.	0.30 (0.03)	0.05 (0.01)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)
C.C.	0.00	0.00	0.00	0.00	0.00
D.J.	0.00	0.00	0.00	0.00	0.00
E.K.	0.50 (0.11)	0.10 (0.02)	0.01 (0.02)	0.10 (0.02)	0.10 (0.02)
M.A.	(0.10) (0.05)	0.10 (0.05)	0.10 (0.05)	0.10 (0.05)	0.10 (0.05)
J.M.	(0.40) (0.07)	0.02 (0.01)	0.00	0.00	0.00
F.S.	(0.30) (0.04)	0.00	0.00	0.00	0.00
S.C.	0.00	0.00	0.00	0.00	0.00

SE, standard error.

Table 2. Erythrocytes Suspended in Autologous Plasma. Erythrocyte Adherence to Cultured Endothelium of Individual Patients

Subjects	Percent Adherence at Various Shear Stresses (SE)				
	1 dyne/cm²	10 dyne/cm²	20 dyne/cm²	30 dyne/cm²	55 dyne/cm²
Sickle cell patients					
I.D.	2.05 (1.12)	0.94 (0.71)	0.89 (0.66)	0.61 (0.61)	0.56 (0.55)
A.T.	1.37 (0.29)	0.90 (0.19)	0.32 (0.03)	0.23 (0.02)	0.09 (0.01)
T.J.	18.00 (4.00)	10.00 (5.00)	7.50 (5.50)	5.50 (4.50)	1.50 (1.50)
A.H.	2.09 (1.13)	0.83 (0.49)	0.83 (0.49)	0.58 (0.33)	0.33 (0.16)
Sickle-thalassemia patients					
P.H.	0.20 (0.05)	0.00	0.00	0.00	0.00
R.M.	2.25 (0.18)	0.92 (0.12)	0.84 (0.10)	0.67 (0.10)	0.50 (0.10)
Controls					
B.S.	1.58 (0.21)	0.87 (0.13)	0.61 (0.09)	0.43 (0.07)	0.12 (0.02)
C.S.	1.70 (0.33)	0.39 (0.12)	0.34 (0.10)	0.22 (0.06)	0.09 (0.02)
A.M.	1.11 (0.14)	0.50 (0.08)	0.36 (0.05)	0.27 (0.03)	0.19 (0.03)

given for individual patients in Table 1 and depicted as the mean percent adherent cells *v* shear stress in Fig 2. The experiment in each case started 30 minutes after the erythrocytes in buffer were allowed to settle on the cultured surface, and since study of each cell required approximately three minutes, the total exposure time of each subsequent cell increased by approximately three minutes over the predecessor, ie, the exposure time of cells was not uniform. However, there was no trend in the data to suggest that increasing pressures were required to detach cells studied late in the experiment, and thus, no consideration for the point in time of study of specific cells was given in the assembly and interpretation of the data. The variation of the frequency of control cell adherence to differing cultures of endothelial cells was less than 1% at the lower shear rates in the case of buffer suspensions and less than 2.0% in plasma; thus data have been expressed as a percentage of the control or experimental cells adherent at each shear stress rather than in the ratio of experimental to control cell or in normalized form.

With gradually increasing shear stress, some sickle cells remained attached to the endothelial cells when subjected to calculated shear forces above the physiologic range as indicated by data from animal models. These persistently attached cells were located on the endothelial cell surface only, and thus the behavior appeared to reflect erythrocyte–endothelial cell surface interaction. Care was taken to avoid cells in regions of detectable surface artifacts since surface abnormality, eg, exposed glass surfaces, could explain resistance to detachment. Adherent cells that required large shear forces for separation frequently reattached instantly upon recontact with the endothelial surface, and equally large shear forces were necessary to separate cells from subsequent readhesions. More than 95% of the adherent cells from sickle cell patients were non-ISC. The ISC that were adherent required lower shear stress for separation from the endothelium than the non-ISC.

When erythrocytes were suspended in autologous plasma instead of buffer and albumin, adherence increased as shown in Table 2. The increased retention by the endothelial surface secondary to plasma was most pronounced in the low and intermediate shear stress ranges. For the three sickle cell patients not in vaso-occlusive crisis, the mean adherence in plasma was the same as the adherence in buffer with albumin

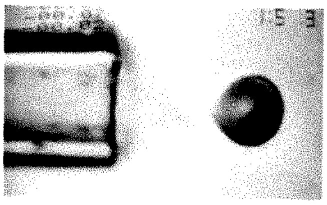

Fig 1. Photograph from a video monitor of an erythrocyte separating from a confluent endothelial monolayer in response to shear stress applied via a fluid-filled micropipette.

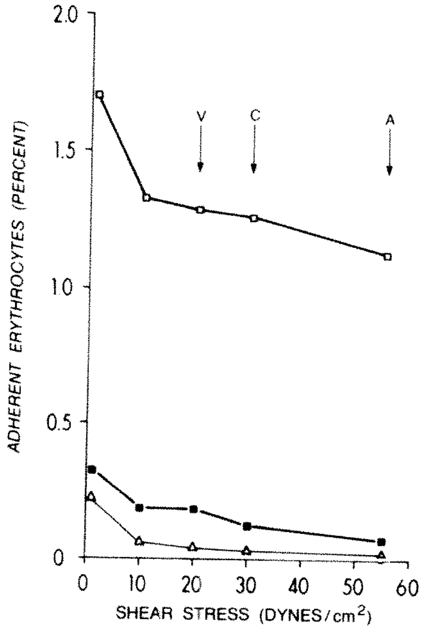

Fig 2. Mean percentage of erythrocytes suspended in isotonic buffer plus albumin that are adherent to cultured endothelial monolayers at various shear stresses. Key: V, maximum venule shear stress; C, maximum capillary shear stress; A, maximum arteriole shear stress; heavy solid line with open squares, hemoglobin S erythrocytes; heavy solid line with closed squares, sickle-thalassemia erythrocytes; light solid line, control erythrocytes.

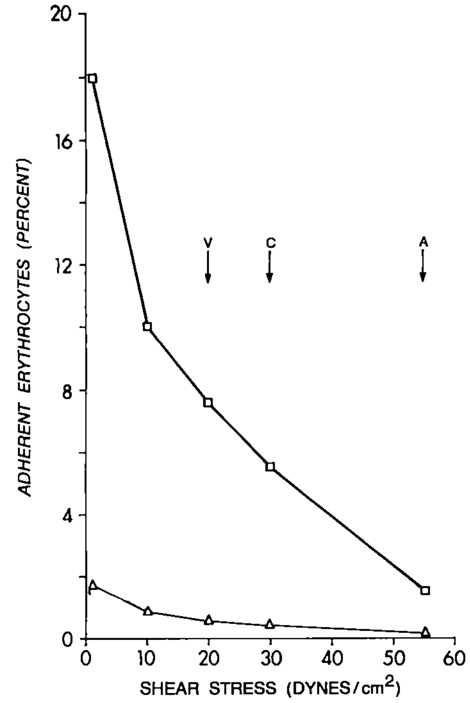

Fig 3. Percentage of erythrocytes suspended in autologous plasma that are adherent to cultured endothelial monolayers at various shear stresses. Key: heavy solid line, sickle crisis patient T.J.; light solid line, control patient B.S.

and resembled adherence of control cells in plasma. This implies that an intrinsic cellular abnormality promotes sickle cell endothelial adherence in buffer and albumin, and this abnormality is not normally influenced by plasma unless a vaso-occlusive crisis occurs. For patient T.J., who was in vaso-occlusive crisis, the presence of autologous plasma caused adherence to increase by more than two orders of magnitude. This is depicted in Fig 3, which is a representative experiment comparing cell adherence in a sickle patient with vaso-occlusive crisis with a control, each cell sample in autologous plasma. Addition of sickle crisis plasma to erythrocytes from a sickle patient not in crisis caused adherence of 12% at low shear stress with a progressive decrease to 1% adherence at maximum shear stress. When autologous plasma is incubated at 60 °C for one hour, it no longer augments adhesion. In studies of sickle and control cells, the presence of 600 mg/100 mL fibrinogen did not alter adherence. Since plasma pH might be expected to alter cell surfaces and plasma protein conformation, the effect of pH on erythrocyte adherence was studied. Variation of plasma or buffer pH over the range 6.5 to 8.0 did not influence retention of erythrocytes by the cultered cell surface (Table 3).

Adherence to surfaces beneath the endothelium exposed by potential breaches in the endothelial monlayer was studied. For control or sickle cells that were not washed, adherence to plastic or glass was nearly identical to adherence to endothelium. However, for washed cells there was a marked difference in adherence. Virtually 100% of washed cells resuspended in buffer and albumin became permanently adherent to glass or plastic surfaces, whereas adherence to endothelium was not affected by washing cells. This adherence was of such magnitude that the membrane remained attached even though the cell was destroyed by high shear force. When washed cells were reexposed to autologous plasma and suspended in buffer and albumin, only 3% demonstrated this irreversible adherence. These studies reemphasize the fact that the wash procedure may remove plasma protein from the erythrocyte surface and thus may contribute to behavior not expected of cells bathed in plasma proteins in the in vivo situation. Of interest, permanent adherence of washed cells to glass is eliminated by preexposing the glass or plastic to the supernatant from endothelial cultures. This suggests that potential breaches in the monolayer covering do not affect adherence characteristics and that endothelial cell products may play a role in modulating erythrocyte–endothelial cell interactions.

DISCUSSION

The key observation of the study is the demonstration that some sickle erythrocytes remain adherent to the cultured endothelial surface at shear stresses greater than those expected in the in vivo circulation at regions where obstructive phenomena occur. Since these experiments were performed in the absence of platelets, the observation supports the earlier interpretation[2] that, in addition to intrinsic rheologic abnormalities of non-ISC, their tendency to adhere to endothelium may contribute as well to initiation of obstruction. It is consistent with the conclusions of Kakukcelebi et al[17] in studies of umbilical cord vessels perfused with sickle cells. Based on these data, increased adhesion in the low–flow rate venous portion of the circulation would be likely, a suggestion that fits the concept of venous obstruction as either an actual one, the result of a rheologic plug of erythrocytes and platelets, or rheologically functional obstruction resulting from cell adherence to the venous wall and increased cell-to-cell interactions that increase resistance to flow. This also fits the prediction of Hochmuth et al[18] that erythrocyte–endothelial cell adherence would be most feasible in regions of intermittent low shear stress.

Table 3. Control Erythrocyte Adherence to Cultured Endothelium as a Function of pH at Various Shear Stresses

	Percent Adherence at Various Shear Stresses*				
pH	1 dyne/cm^2	10 dyne/cm^2	20 dyne/cm^2	30 dyne/cm^2	55 dyne/cm^2
7.37	0.16	0.04	0 03	0 03	0 02
7.20	0.15	0.03	0 03	0 03	0.02
6.98	0.16	0.04	0 03	0 03	0.03
6.50	0 04	0.01	0 01	0.01	0.00

*Minimum of 1,000 cells observed for each shear stress.

Approximately 5% of sickle cell patients experience occlusive episodes involving the CNS[19]; these correlate with large-vessel arterial disease.[20] In the high-velocity portions of the circulation, increased frequency of sickle erythrocyte movement from the axial stream to slower movement in the plasma layer adjacent to the endothelium might offer opportunity for adhesion; however, vascular abnormalities augmenting such cell movement to the vessel surface and also the presence of platelets probably are necessary for initiation of thrombous formation.

An important observation is the enhancement of erythrocyte–endothelial cell adhesion by the presence of plasma; this is similar to the studies of Mohandas and Evans.[13] The greater influence of plasma from blood obtained during sickle crisis clearly supports the earlier conclusion of Hebbel et al[11] from in vitro studies of sickle cell adherence to cultured endothelial cells.

The specific basis for the abnormal retention of sickle cells by the cultured endothelial cells is not clear. Earlier suggestions that sickle cells were deficient in sialic acid, the principle determinant of surface charge, have not been confirmed,[21] and the suggestion that the triggering feature is an abnormal distribution of sialic acid on the membrane surface has not been sustained by experiments.[22] Thus, although the phenomenon must involve the erythrocyte's outer membrane surface, definition of a molecular mechanism to explain the avidity is not yet available. It is of interest that pH has no important effect, since pH change probably influences binding of plasma proteins to the cell surface. Attachment in these experiments occurs during the 30-minute incubation period when erythrocytes are resting on the endothelial surface, and during this interval in which the two membrane surfaces are very closely approximated, many biochemical processes may occur, the net result of which is to effect attachment. Although the initiating factors of the surface-to-surface interaction are not obvious, it is of interest that many adherent cells, after being separated from the endothelium, immediately reattach, suggesting that the significant phase of this interaction occurs very early in the contact of the cell surfaces or that the prior changes render the cells more liable to reattachment

These studies may be interpreted to suggest that both the features of the sickle cell surface and plasma contribute to significant erythrocyte–endothelial cell adherence in sickle cell anemia. As reasoned by Hebbel et al[12] the plasma factors may be the modulator of adherence in the pathophysiologic process, since erythrocyte membrane surface characteristics would be presumed to be relatively constant after the reticulocyte stage. Reduced sickle cell deformability leading to relative obstruction or slowing of flow, membrane characteristics predisposing to adherence, and plasma factors may combine to produce the vascular phenomena typical of the disease. It seems probable that flow must be slowed sufficiently or local flow dynamics modified to produce opportunity for the sickle cell–endothelial cell contact leading to adhesion. This latter suggestion is supported by the observation of Burns et al[14] that vortex flow promotes significant erythrocyte–endothelial cell adherence for sickle cells.

REFERENCES

1. Hoover R, Rubin R, Wise G, Warren: Adhesion of normal and sickle erythrocytes to endothelial monolayer cultures. Blood 54:872, 1979

2. Hebbel RP, Yamada O, Moldow CF, White JG, Eaton JW: Abnormal adherence of sickle erythrocytes (HbS RBC) to vascular endothelium. Blood 52:98, 1978 (suppl 1)

3. Chien S, Usami S, Bertles JF: Abnormal rheology of oxygenated blood in sickle cell anemia. J Clin Invest 49:623, 1970

4. Richardson SG, Matthews KB, Stuart J, Geddes AM, Wilcox RM: Serial changes in coagulation and viscosity during sickle cell crisis. Br J Haematol 41.95, 1979

5. Kenny MW, Meakin M, Worthington DJ, Stuart J Erythrocyte deformability in sickle cell crisis. Br J Haematol 49 103, 1981

6 Cokelet GR: Macroscopic rheology and tube flow of human blood, in Grayson J, Zingg W (eds): Microcirculation, vol 1. New York, Plenum, 1976, p 9

7. Zweifach BW, Lipowsky HH: Quantitative studies of microcirculatory structure and function. Circ Res 41:380, 1977

8. Cokelet GR: The rheology of human blood, in Fung YC, Perrone N, Anliker M (eds)· Biomechanics, Its Foundations and Objectives. Englewood Cliffs, NJ, Prentice-Hall. 1972, p 63

9 Goldsmith HL, Mason SG· The microrheology of dispersions, in Eirich FR (ed): Rheology—Theory and Applications, vol 5. Orlando, Fla, Academic Press, 1967, p 85

10. Hebbel RP, Boogaerts MA, Eaton JW, Steinberg MH: Erythrocyte adherence to endothelium in sickle-cell anemia. N Engl J Med 302:992, 1980

11. Hebbel RP, Yamada O, Moldow CT, Jacob HS, White JG, Eaton JW: Abnormal adherence of sickle erythrocytes to cultured vascular endothelium Possible mechanism for microvascular occlusion in sickle cell disease. J Clin Invest 65:154, 1980

12 Hebbel RP, Moldow CF, Steinberg MH: Modulation of erythrocyte-endothelial interactions and the vaso-occlusive severity of sickling disorder. Blood 58:947, 1981

13. Mohandas N, Evans E: Adherence of sickle erythrocytes to vascular endothelial cells: Requirement for both cell membrane changes and plasma factors. Blood 64:282, 1984

14 Burns ER, Wilkinson WH, Nagel RH. Adherence properties of sickle erythrocytes in dynamic flow systems. J Lab Clin Med 105:673, 1985

15. Lipowsky HH, Kovalcheck S, Zweifach BW. The distribution of blood rheological parameters in the microvasculature of cat mesentery. Circ Res 43:738, 1978

16 Klitzman B, Duling BR: Microvascular hematocrit and red flow in resting and contracting striated muscle. Am J Physiol 237:481, 1979

17 Kakukcelebi A, Barmaloski SP, Barnhart MI. Interactions between vessel wall and perfused sickle erythrocytes Scan Electron Microsc 3:243, 1980

18. Hochmuth RM, Mohandas N, Spaeth EE, Williamson JR, Blackshear PL, Johnson DW: Surface adhesion, deformation and detachment at low shear of red cells and white cells. Trans Am Soc Artif Intern Organs 18:325, 1972

19 Powars D, Wilson B, Imbus C, Pegelow C, Allen J· The natural history of stroke in sickle cell disease. Am J Med 65·461, 1978

20. Powars D, Imbus C. Cerebral vascular accidents in sickle cell anemia. Tex Rep Biol Med 40:293, 1980

21. Riggs MG, Ingram VM: Differences in erythrocyte membrane proteins and glycoproteins in sickle cell disease. Biochem Biophys Res Commun 74.191, 1977

22 Clark LJ, Chan LS, Powars DR, Baker RF· Negative charge distribution and density on the surface of oxygenated normal and sickle red cells. Blood 57:675. 1981

Variables Influencing the Timing of Marrow Transplantation in Patients With Chronic Myelogenous Leukemia

By George B. Segel, William Simon, and Marshall A. Lichtman

The prognosis for patients with chronic myelogenous leukemia (CML) has improved only for patients who can receive marrow transplantation from a histocompatible sibling. The timing of the marrow transplant is made difficult by the high peritransplant mortality of 20% to 35% and a group of patients with a prolonged chronic phase of CML, which can be identified on the basis of prognostic indexes (age, percent blood myeloblasts, spleen size, and platelet count). We have developed a mathematic model and computer program that consider age, prognostic index, and projected survival rate by transplantation to balance the risk of peritransplant mortality against the risk of delaying the transplantation of patients with Philadelphia chromosome–positive CML. The computation assesses the risk of delaying transplantation; it does not offer the option of avoiding transplantation, since long-term survival ultimately requires transplantation. Three prognostic groups were considered as described by Sokal and co-workers (Blood 63:789, 1984) (I, best; II, intermediate; III, worst prognosis). The computation used the projected survival rates of transplantation from the Seattle experience and from the International Bone Marrow Transplant Registry. As an example of the model's utility, we have determined the ratio of the calculated life expectancy to the normal life expectancy for hypothetical patients up to 50 years of age in each of the three prognostic categories. A value of 20% is used for patients who successfully receive transplants after the onset of the accelerated phase. The analysis allows assessment of the risk of delaying transplantation for a finite time in patients with CML. The importance of the method rests in its consideration of multiple variables, including the peritransplant mortality, transplant projected survival before and upon entering the accelerated phase, age, prognostic group, and other risk factors. The program permits a change in these parameters as new information or advances in treatment occur. This analysis does not replace the diagnostic deliberations of the clinician. Rather, it provides a numeric framework for prognosis based on the currently available data. The physician in conjunction with the patient, not the algorithm, makes the decisions of whether and when to transplant.

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THE LIFE EXPECTANCY of the patient with chronic myelogenous leukemia (CML) treated with chemicals or radiation has not changed substantially over the past 50 years. Although median survival has increased by 6 months to a year as a result of better medical treatment, the disease progresses to a highly lethal accelerated form in nearly all cases. Half of the patients with CML die within about 3 years and more than 70% within about 5 years from the time of diagnosis.[1]

The advent of marrow transplantation from allogeneic sibling donors has led to an increase in the proportion of patients with CML surviving beyond 3 years. The decision of when to recommend marrow transplantation is made difficult, however, by the high peritransplant mortality of 20% to 35% and the ability to transplant successfully a significant proportion of patients (10% to 30%) after they enter the accelerated phase of the disease.[1-4] In addition, the ability to identify patients who are likely to have a prolonged chronic phase of their disorder based on several prognostic indexes at the time of diagnosis makes the decision about when to recommend marrow transplantation even more complex.[5-7]

In this report we have developed a model that considers age, prognostic indexes, and the projected survival with transplantation in order to balance the risk of peritransplant mortality against the risk of delaying transplantation for a patient with Philadelphia chromosome (Ph^1) positive CML.

MATERIALS AND METHODS

Calculation of Chance of Survival

Basic calculation of chance of survival This analysis determines the chance of survival from the time of diagnosis, that is, the proportion of the original population that achieves a normal life span if marrow transplantation is performed at some time after diagnosis. Figure 1 illustrates a hypothetical survival curve [S(t)] of patients with CML. The horizontal axis indicates years after diagnosis, and the vertical axis indicates the surviving fraction of the original population of patients In this example approximately 60% of the patients [$S(T_1)$] survive 4 years without transplantation, and the probability of surviving 4 years without transplantation is therefore approximately 0.6.

If at 4 years, the patient is still in the chronic phase of CML, has a histocompatible donor, and receives a marrow transplant, there is, for example, a 70% chance of long-term survival or cure after transplantation, ie, K = 0.7 The combined probability of surviving CML for 4 years and the transplantation procedure at the end of the fourth year equals the product of the survival fraction of the transplant (K) and the fractional survival at 4 years [$S(T_1)$]; thus, $KS(T_1) = 0.7 \times 0.6 = 0.42$ or 42%.

Correction for salvage transplantation during the accelerated phase prior to T_1. A fraction of patients, L, who develop accelerated disease during the 4-year period can be salvaged by marrow transplantation at the time that CML progresses. Up to T_1 the fraction of the original population that develops accelerated disease is [1−S(t)], and at T_1 it is [$1-S(T_1)$] (Fig 2). Thus, if L = 0 3 or 30% and $1 - S(T_1) = 0\ 4$, then the product of $1 - S(T_1)$ and L or 0.12 should be added to the survival of patients who received transplants sucessfully at 4 years, making the chance of survival for all CML patients with a marrow donor 54% Thus, the chance of survival calculation, which includes both elective transplantation at

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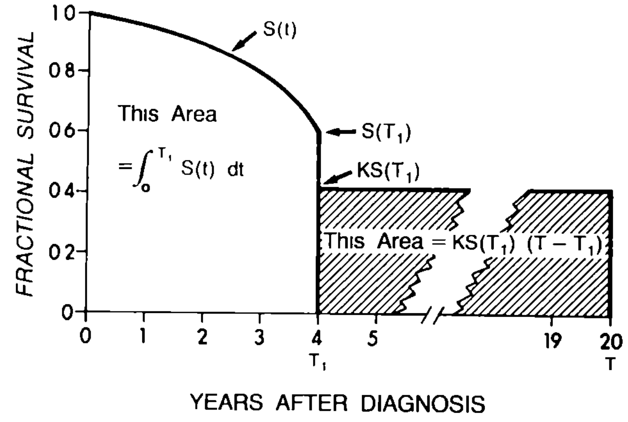

Fig 1. Calculation of the chance of survival and mean life expectancy without salvage. This Figure represents a hypothetical survival curve designated S(t) for patients with CML. In this example, bone marrow transplantation is performed at time T_1 when the fractional survival from the disease is 0.6. The combined probability of surviving CML for 4 years and the transplantation procedure is $KS(T_1)$. The chance of survival is equal to $KS(T_1)$ plus $L[1 - S(T_1)]$, which represents the correction for salvage transplantation during the accelerated phase of the disease prior to T_1 (Fig 2). The mathematical representation of the area under each portion of the survival curve is necessary for the calculation of the mean life expectancy. The derivation of these calculations is presented in the text.

time T_1 and transplantation if accelerated disease develops before T_1, is as follows:

$$\text{Chance of survival beyond } T_1 = KS(T_1) + L\,[1 - S(T_1)]. \quad (1)$$

Calculation of Mean Life Expectancy

Basic calculation of mean life expectancy The calculation of mean life expectancy, that is, the product of the proportion of patients cured and the number of surviving years, provides an alternative index for prognosis and timing of marrow transplantation We have chosen 70 years of age as the normal life span, and T is equal to 70 years minus the patient's age. To calculate the life expectancy with transplantation at time T_1, we compute the area under the curve in Fig 1 up to time T_1 Added to this quantity is the area under the curve equal to the transplant survival fraction, $KS(T_1)$ multiplied by $T - T_1$. Thus,

$$\text{Life expectancy (years)} = \int_0^{T_1} S(t)dt + KS\,(T_1)\,(T - T_1). \quad (2)$$

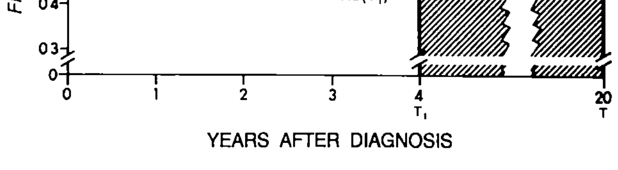

Fig 2. Calculation of the chance of survival and mean life expectancy with salvage. This Figure is similar to Fig 1 and represents a hypothetical survival curve designated S(t). The mean life expectancy is the product of the proportion of patients cured and the number of surviving years. Thus, the life expectancy with transplantation at time T_1 is equal to $\int_0^{T_1} S(t)dt$ plus $KS(T_1)$ $(T - T_1)$. The survival curve is corrected for the proportion of patients who are salvaged by transplantation during the accelerated phase of the disease prior to T_1. The corrected curve is designated $S'(T_1)$. The derivation of the calculation for mean life expectancy incorporating the salvage transplantation during the accelerated phase of CML is described in the text.

The calculation of the integral up to T_1 is most conveniently done by drawing a smooth curve through the survival data points (without transplantation) and reading the height of the curve at small, equal time intervals. The area within an interval is the width of the interval multiplied by the average of the height on the left and right edges of the interval. The height of the right edge of the interval ending at T_1 is $S(T_1)$ (Fig 3).

Correction for salvage transplantation during accelerated phase prior to T_1. Life expectancy from the time of diagnosis to T_1 has been calculated for the various values of T and T_1 In this example we assume that patients survive to T_1 in the chronic phase of CML If, however, the disease accelerates during this time, there is still the possiblity of a cure using marrow transplantation.[2-4] This survival fraction, L, should be included in the calculation of life expectancy The fraction of patients in whom accelerated disease develops up to time t, within the time T_1, is $[1 - S(t)]$ (Fig 2). At any point in time t up to T_1, those patients in whom accelerated disease has developed could receive transplants with a probability of success, L, or a probability of failure, $(1 - L)$ Thus, the survival curve $S(t)$ shown in Figs 1 and 2 is modified as shown by the dotted line $S'(t)$ in Fig 2 in which the nonsurviving fraction $1 - S(t)$ is multiplied by the factor $1 - L$, and thus the surviving fraction is increased. Note that the quantity $1 - L$ indicates that if L is equal to 0 there are no successful transplants during the accelerated phase of the disease and there is no change in the survival curve. If L equals 1, then $1 - L$ equals 0, and all of the patients in an accelerated phase would receive transplants successfully, and the new survival curve (dotted lines) would be a horizontal line at the survival fraction equal to 1. In this example, the value for L is approximately 0 3; that is, 30% of those

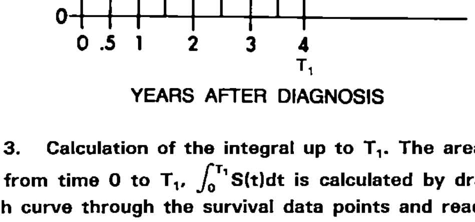

Fig 3. Calculation of the integral up to T_1. The area of the curve from time 0 to T_1, $\int_0^{T_1} S(t)dt$ is calculated by drawing a smooth curve through the survival data points and reading the height of the curve at small time intervals. The area within an interval is the width of the interval multiplied by the mean height of the left and right edges of the interval. The height of the right edge of the interval ending at T_1 is $S(T_1)$. The area under the curve after the time of bone marrow transplantation T_1 is equal to $KS\,(T_1)\,(T - T_1)$ (see text).

patients entering an accelerated phase can be salvaged by treatment with marrow transplantation

The possibility of long-term survival after transplantation during the accelerated phase of disease increases both the chance of survival (see the preceding material) and the calculated life expectancy.

To evaluate life expectancy:

Replace $S(t)$ in the integral of equation 2 by:

$$S'(t) = 1 - [1 - S(t)]\,(1 - L) = S(t) + L[1 - S(t)] \qquad (3)$$

and add $[S'(T_1) - S(T_1)](T - T_1)$, which is the increment in survival rate resulting from transplantation in the accelerated phase.

$$\text{Life expectancy} = \int_0^{T_1} S'(t)dt + [KS(T_1) + S'(T_1) - S(T_1)](T - T_1) \qquad (4)$$

$$\int_0^{T_1} S'(t)dt = \int_0^{T_1} \{S(t) + L[1 - S(t)]\}dt = LT_1 + (1 - L)\int_0^{T_1} S(t)dt \qquad (5)$$

$$KS(T_1) + S'(T_1) - S(T_1) = KS(T_1) + L[1 - S(T_1)] \qquad (6)$$

$$\text{Life expectancy} = LT_1 + (1 - L)\int_0^{T_1} S(t)dt + (T - T_1)\,[L + (K - L)S(T_1)] \qquad (7)$$

$$\text{Life expectancy (years)} = LT + (K - L)S(T_1)\,(T - T_1) + (1 - L)\int_0^{T_1} S(t)dt. \qquad (8)$$

This latter result appears complicated because the acute-phase transplantation can occur at any time within T_1 and because the factor L already includes the transplantation risk. However, the computation is easily done using the computer program listed in the attached Appendix.

RESULTS AND DISCUSSION

We have established a mathematic analysis in which two prognostic indexes can be calculated for a group of patients with Ph^1-positive CML: the chance of survival, ie, chance of living a normal life span, and the mean life expectancy for a CML patient. Without marrow transplantation, life expectancy is limited, and patients die at rates shown in Fig 4 for each of three prognostic groups (see the following).[7] For those patients with a histocompatible donor, marrow transplantation provides the potential for cure. The object of this analysis is to permit a more precise assessment of how long a patient can delay transplantation without incurring undue risk. The mathematic calculation considers the age of the patient, the prognostic category of the patient's disease, the projected survival rate for allogeneic marrow transplantation, and the projected salvage of patients by transplantation in whom accelerated disease develops.

Calculated Life Expectancy

All eligible patients with CML require transplantation to permit the possibility of normal life expectancy. The projected survival for allogeneic marrow transplantation in CML patients taken from data published by the Seattle group is 55%,[4] by the UCLA group is 70%,[2] and by the International Bone Marrow Transplant Registry is 65%.[3] These results include patients from 5 to 50 years of age without regard for prognostic factors. The projected salvage rates of patients transplanted after developing accelerated disease or blast crisis are, respectively, 15% and 15% in the Seattle study,[4] 35% and 10% in the UCLA study,[2] and 40% and 10% in the International Bone Marrow Transplant Registry study.[3]

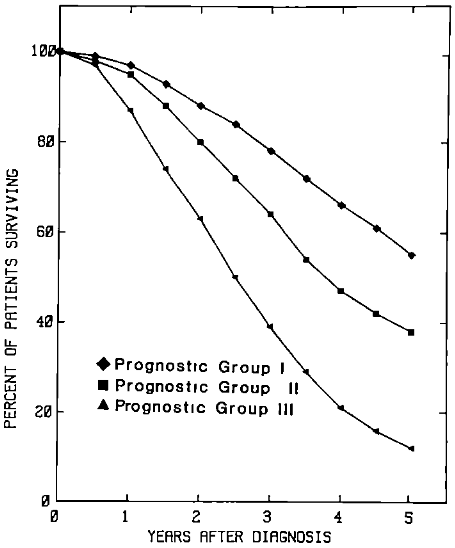

Fig 4. **Survival curves of CML according to prognostic group without transplantation. The percentage of patients with CML who survive at each time up to 5 years after diagnosis is shown for each of three prognostic groups as defined by Sokal et al.[7] The designation of the prognostic group depends upon the age of the patient, the spleen size, the platelet count, and the percentage of myeloblasts in the blood.**

In the following analysis we have determined the calculated life expectancy for a cohort of 20-year-old patients, assuming a 65% projected survival after transplantation in chronic phase and a 20% projected survival after transplantion in the accelerated or blastic phase of CML as being representative of the published studies.[2-4]

The maximum calculated mean life expectancy is 0.65 of the normal life expectancy of a 20-year-old, in this case the remaining 50 years (70 − 20 years). Thus the calculated mean life expectancy is 32.5 years if the patients receive transplants at the time of diagnosis (Fig 5). This calculated life expectancy decreases as transplantation is delayed from the time of diagnosis (lower curve). When the calculation is adjusted for a 20% projected survival for patients who received transplants during the accelerated phase or blast crisis, the overall slope of the curve is more shallow (Fig 5).

Table 1 shows the data for the upper curve of Fig 5, ie, calculated life expectancy with a 65% projected survival after transplantation in the chronic phase and a 20% projected survival with transplantation in the accelerated phase of CML. We have calculated two types of prognostic curves from these data to assess the impact of delay in transplantation. The first is a ratio of the calculated mean life expectancy to the normal life expectancy (CLE/NLE) expressed as a percentage (Table 1). The second is the chance of survival, which is calculated at a given time as the sum of the proportion of patients "cured" by allogeneic transplantation during chronic and accelerated phases of CML. The initial

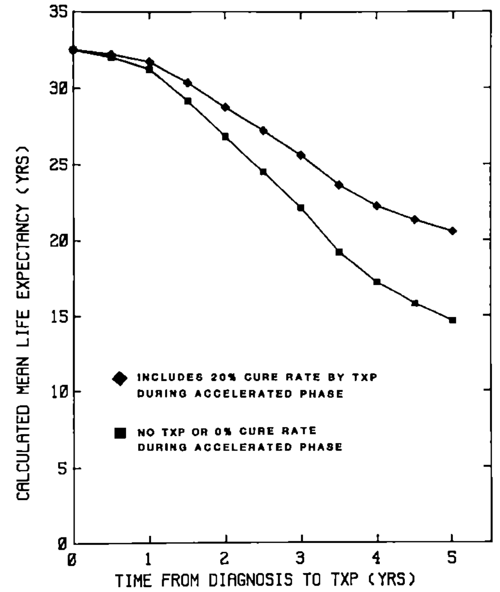

Fig 5. Calculated mean life expectancy without and with a 20% rate of salvage for transplant recipients during the accelerated or blastic phases of CML. The calculated mean life expectancy is shown for a cohort of 20-year-old patients with a 65% survival after bone marrow transplantation and a 20% salvage rate for transplantation during the accelerated or blastic phase if this occurs prior to the time chosen for transplantation, T_1. The initial point on the curve, 32.5 years, represents the maximal life expectancy if transplantation is performed at the time of diagnosis. In this case it is 0.65 of the normal life expectancy of a 20-year-old, ie, the remaining 50 years (70 − 20).

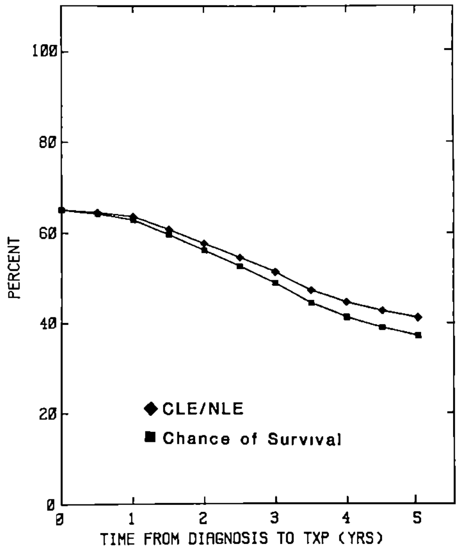

Fig 6. CLE/NLE ratio and chance of survival. These prognostic curves assess the impact of delay from the time of diagnosis to bone marrow transplantation on the prognosis in patients with CML who have a histocompatible donor. The curves represent data for a cohort of 20-year-old patients with a 65% survival after transplantation in the chronic phase and a 20% success of transplantation in the accelerated or blastic phase of CML. The CLE/NLE is the ratio of the calculated mean life expectancy to the normal life expectancy expressed as a percentage. The chance of survival is calculated at a given time as the sum of the proportion of patients cured by allogeneic transplantation during the chronic and accelerated phases of CML.

point on these curves, .65, is both the CLE/NLE ratio and the chance of survival if transplantation is performed at the time of diagnosis. The curves are similar, especially in the early phase of the disease (Fig 6). They diverge if transplantation is delayed, and the divergence is greater in older patients (not shown). The chance of survival is less favorable than the CLE/NLE ratio because it does not include the survival time prior to transplantation. Importantly, the curves are nearly flat during the first year after diagnosis, and there is a negligible risk of delaying the transplant for 1 year in this hypothetical patient.

Table 1. Calculated Life Expectancy for 20-Year-Old Patients in Prognostic Group 2 With a 65% Projected Survival After Transplantation in Chronic Phase and a 20% Projected Salvage After Transplantation in Accelerated Phase or Blast Crisis

Transplantation Time	Calculated Life Expectancy (yr)	CLE/NLE (%)	Chance of Survival (%)
0	32.5	65.0	65.0
0.5	32.2	64.4	64.1
1	31.7	63.4	62.7
1.5	30.3	60.7	59.6
2	28.7	57.5	56.0
2.5	27.1	54.3	52.4
3	25.5	51.1	48.8
3.5	23.5	47.1	44.3
4	22.2	44.4	41.1
4.5	21.2	42.5	38.9
5	20.5	41.0	37.1

Influence of Prognostic Group

A variety of variables has been correlated with prognosis in CML.[5–18] The data supporting an association between historic factors and prognosis are weak.[7,9,10,12] However, there is substantial support for liver and spleen size,[5–7,11–13] myeloblasts in the blood[5,7,12,13] and bone marrow,[6,7,12] basophilia,[7,12,13] and thrombocytopenia or thrombocytosis[5,7,10–13,16,17] as indicators of prognosis. The Philadelphia chromosome[6,7,9,10,12,13] and myelofibrosis[12–15,18] also are prognostic findings.

Tura and his co-workers established the relationship of six clinical features to overall prognosis.[5] These include spleen size, liver size, blood progranulocytes and myeloblasts, total white count, and platelet count. Three prognostic groups were established from these data. The best prognostic group had no or one negative prognostic feature, the intermediate group had two or three negative prognostic features, and the worst prognostic group had four or more negative prognostic features.[5] These data were developed from two series of patients with CML, one containing 116 patients and the other containing 139 patients. A similar prognostic grouping was established by Sokal and co-workers, who analyzed data from 813 chronic-phase, Ph^1-positive patients with CML who were studied at six medical centers.[7] In this study the prognostic factors were determined using Cox's proportional hazard model for covariant analysis.[19] Sokal and co-workers developed a hazard ratio function based on the age of the patient, the size of the spleen, the platelet count, and the percentage of blasts in the blood.[7] We have chosen to use

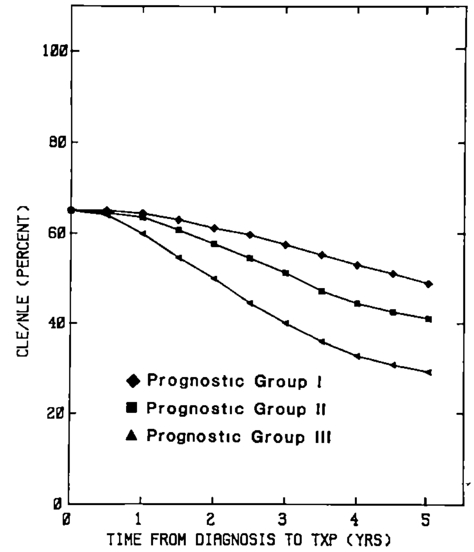

Fig 7. Influence of prognostic group on the CLE/NLE ratio. The CLE/NLE is the ratio of the calculated mean life expectancy to the normal life expectancy expressed as a percentage. In this analysis the prognostic group was determined from a hazard ratio function based on the patient's age, spleen size, platelet count, and the percentage of blasts in the blood as described by Sokal et al.[7] The curves represent data for a cohort of 20-year-old patients with a 65% survival after transplantation in the chronic phase and a 20% success of transplantation in the accelerated or blastic phase of CML.

Sokal and co-workers' calculation of the hazard ratio function so that the prognostic group can be determined from the patient characteristics. This calculation is incorporated into the computer program.

Evaluation of a 20-year-old patient with chronic-phase CML is shown in Fig 7. The projected survival after marrow transplantation is 65%, and the projected salvage by transplantation in the accelerated phase is 20%. The ratio CLE/NLE is shown for the three prognostic groups. The initial points represent transplantation at the time of diagnosis and cannot be greater than the projected survival for transplantation in the chronic phase, ie, 65%. For the first and second prognostic groups the analysis indicates that the CLE/NLE ratio is unchanged and thus marrow transplantation could be delayed for 1 year with little additional risk. In contrast, the CLE/NLE ratio for patients in the third prognostic group begins to fall sharply 6 months after the time of diagnosis. This analysis indicates that the least risk would be incurred if marrow transplantation was performed immediately for patients in the worst prognostic group.

Influence of Patient Age

Younger patients fare better after marrow transplantation. Of 34 transplant recipients under the age of 30 in Seattle, the projected survival was 58%, whereas of 33 patients over the age of 30 years, the projected survival was 40%.[4] In patients accumulated from 36 centers by the International Bone Marrow Transplant Registry, projected survival was 82% in 14 patients under 25 years of age and 52% in 25 patients over 25 years of age.[3] We have determined the CLE/NLE ratio for two hypothetical patients aged 20 and 40 years in each of the three prognostic categories using the percent survival by age group from Seattle and the International Bone Marrow Transplant Registry (Figs 8 and 9). A value of 20% was used in this analysis for the salvage of patients who were transplanted after onset of the accelerated phase.

For patients aged 20 years in prognostic categories I and II, a delay of 1 year prior to transplantation posed little additional risk to overall survival, in contrast to those patients in prognostic group III where the CLE/NLE ratio fell at an accelerated rate after 6 months (Fig 8). The result was similar in the analysis of the data from Seattle or the International Bone Marrow Transplant Registry.

For those patients 40 years of age, it was no longer clear that transplantation for group III patients should be undertaken within the first 6 months (Fig 9). In the Seattle series, the CLE/NLE ratio did not decrease significantly until 1 year following diagnosis. The International Bone Marrow

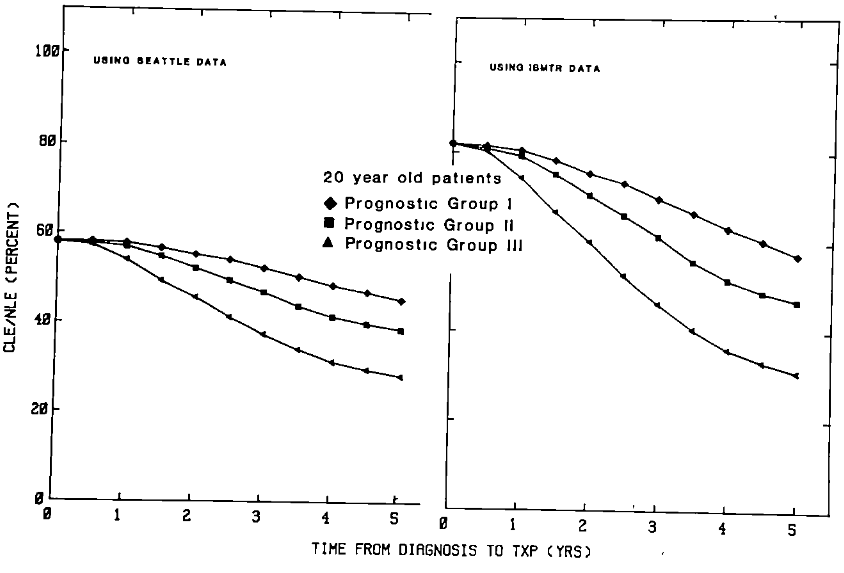

Fig 8. Influence of patient age on the CLE/NLE ratio in a 20-year-old cohort. The CLE/NLE is the ratio of the calculated mean life expectancy to the normal life expectancy expressed as a percentage. In this analysis the CLE/NLE ratio was calculated for the three prognostic groups using the published success rates for transplantation from Seattle and from the International Bone Marrow Transplant Registry (IBMTR). This Figure may be compared with Fig 9, which provides a similar analysis for 40-year-old patients.

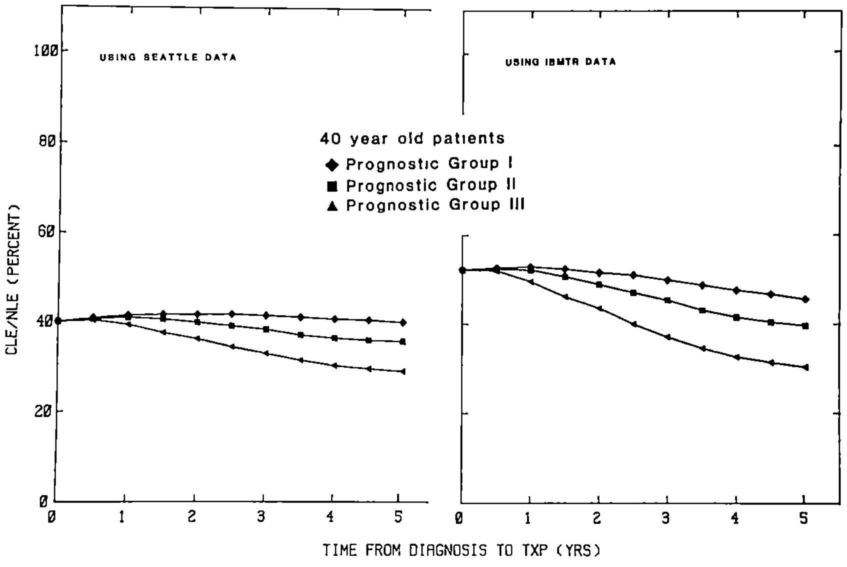

Fig 9. Influence of patient age on the CLE/NLE ratio in a 40-year-old cohort. The CLE/NLE is the ratio of the calculated mean life expectancy to the normal life expectancy expressed as a percentage. In this analysis the CLE/NLE ratio was calculated for the three prognostic groups using the published success rates for transplantation from Seattle and from the IBMTR. This Figure may be compared with Fig 8, which provides a similar analysis for 20-year-old patients.

Transplant Registry series had a higher projected survival for transplantation of older patients (52% *v* 40%). The increased projected survival for transplantation results in a slightly more rapid decrease in the CLE/NLE ratio in group III patients reported by the International Bone Marrow Transplant Registry. Although transplantation should eventually be performed to achieve a normal life span in any of the patients, its delay may be justified when one considers the patient's age and the projected survival with transplantation.

Other Risk Factors

Our analysis is based on the assumption that the delay in transplantation does not diminish the success of the procedure. There is a suggestion in studies performed in Seattle that there may be a decreased efficacy of transplantation as the duration of chronic phase of CML increases.[4] A risk of this type could be incorporated into the analysis if this observation is substantiated. The computer program includes the option for inserting a factor less than 1 to indicate decreased projected survival for transplantation with increasing duration of chronic phase. If, for example, a 1-year delay results in only 80% of the projected survival for a transplantation performed at the time of diagnosis, a factor of 0.8 rather than 1.0 could be entered, and the CLE/NLE ratio would decrease as transplantation is delayed.

A second assumption is that successful transplantation results in a normal life span. Since no data are available, we have used a life span of 70 years for the purpose of analysis. If future clinical studies indicate a shortened life span after transplantation, a value less than 70 years can be entered into the program. We have conducted an analysis at a life span of 60 years for transplant recipients, and little change was observed in the shape of the CLE/NLE ratio curves, although a shortened life span tends to make early transplantation less desirable.

A related assumption is that the projected survival for transplant recipients already incorporates those patients who relapse after transplantation. If late posttransplant relapse reduces the survival further, the new, lower rate can be used in the analysis when this information becomes available. Such late relapse would tend to favor the delay of transplantation.

How To Assess The Computed Data

The model incorporates the impact of a patient's age, prognostic group, and the success rate of transplantation. The program computes a prognostic parameter, the CLE/NLE ratio, which applies to a group of patients with similar characteristics. The CLE/NLE ratio is a summary statement of the risk of delaying marrow transplantation in CML. The ratio thus provides a specific concept that can be used to weigh treatment alternatives, and a common language for assessment of multiple variables by physicians who care for patients with CML.

The information should be used to integrate the observed variables and aid the physician and patient in the decision of when to transplant. The patient's and physician's own personalities and nonmedical factors in the patient's life will also play a role in the timing of transplantation. The program, however, helps the physician and the patient to make an informed decision.

REFERENCES

1. Champlin RE, Golde DW: Chronic myelogenous leukemia Recent advances. Blood 65:1039, 1985

2. Champlin R, Mitsuyasu R, Elashoff R, Gale RP: The role of bone marrow transplantation in the treatment of chronic myelogenous leukemia, in Gale RP (ed): Recent Advances in Bone Marrow Transplantation. New York, Liss, 1983, p 141

3. Speck B, Bortin MM, Champlin R, Goldman JM, Herzig RH, McGlave PB, Messner HA, Weiner RS, Rimm AA: Allogeneic bone marrow transplantation for chronic myelogenous leukaemia. Lancet 665, 1985

4. Thomas ED, Clift RA, Fefer A, Appelbaum FR, Beatty P, Bensinger WI, Buckner CD, Cheever MA, Deeg HJ, Doney K,

Flournoy N, Greenberg P, Hansen JA, Martin P, McGuffin R, Ramberg R, Sanders JE, Singer J, Stewart P, Storb R, Sullivan K, Weiden PL, Witherspoon R: Marrow transplantation for the treatment of chronic myelogenous leukemia. Ann Intern Med 104.155, 1986

5. Tura S, Baccarani M, Corbelli G. Staging of chronic myeloid leukaemia Br J Haematol 47:105, 1981

6. Cervantes F, Rozman C: A multivariate analysis of prognostic factors in chronic myeloid leukemia. Blood 60·1298, 1982

7. Sokal JE, Cox EB, Baccarani M, Tura S, Gomez GA, Robertson JE, Tso CY, Braun TJ, Clarkson BD, Cervantes F, Rozman C· Prognostic discrimination in "Good-Risk" chronic granulocytic leukemia. Blood 63:789, 1984

8 Sjogren U, Brandt L, Mitelman F: Relation between life expectancy and composition of the bone marrow at diagnosis of chronic myeloid leukaemia. Scand J Haematol 12:369, 1974

9. Kardinal CG, Bateman JR, Weiner J: Chronic granulocytic leukemia. Arch Intern Med 136:305, 1976

10. Schilling RF, Crowley JJ: Prognostic signs in chronic myelocytic leukemia. Am J Hematol 7.1, 1979

11. Haanen C, Hellriegel KP, Machin D: E.O R.T.C. Protocol for the treatment of good-risk patients with chronic myelogenous leukemia. A randomized trial. EORTC News 803, 1978

12. Gomez GA, Sokal JE, Walsh D: Prognostic features at diagnosis of chronic myelocytic leukemia. Cancer 47:2470, 1981

13. Jacquillat C, Chastang C, Tanzer T, Weil M, Pereira-Neto M, Gemon-Auclerc MF, Schaison G, Domingo M, Boiron M, Bernard J: Factors de pronostic de la leucemie myeloide chronique. A propos de 798 observations. Nouv Rev Fr Hematol 15:229, 1975

14. Gralnick HR, Harbor J, Vogel C: Myelofibrosis in chronic granulocytic leukemia. Blood 37:152, 1971

15 Clough V, Geary CG, Hashmi K, Davson J, Knowlson T: Myelofibrosis in chronic granulocytic leukaemia. Br J Haematol 42.515, 1979

16. Mason JE, DeVita VT, Canellos GP: Thrombocytosis in chronic granulocytic leukemia: Incidence and clinical significance. Blood 44:483, 1974

17. Monfardin S, Gee T, Fried J, Clarkson B. Survival in chronic myelogenous leukemia: Influence of treatment and extent of disease at diagnosis. Cancer 31:492, 1973

18. Buyssens N, Bourgeois NH: Chronic myelocytic leukemia versus idiopathic myelofibrosis· A diagnostic problem in bone marrow biopsies. Cancer 40·1548, 1977

19. Cox DR: Regression models and life tables J R State Soc (Series B) 34.187, 1972

APPENDIX

Program Listing of CMLPROG BAS

```
10     REM CMLPROG.BAS: CALC OF BOTH CLE/NLE AND CHANCE OF SURVIVAL
15     WIDTH 80
20     DIM S(10, 3): SCREEN 0, 0, 0  COLOR 7, 1, 1· KEY OFF  CLS
30     DIM TXP (20), CALC (20), CLE (20), CHANCE (20)
40     PRINT  PRINT  PRINT: PRINT: PRINT
50     PRINT "    **************************************************
60     PRINT "    ******                                      ******
70     PRINT "    ****** TIMING OF BONE MARROW TRANSPLANTATION IN CML ******
80     PRINT "    ******                                      ******
90     PRINT "    ******  G.B. SEGEL   W. SIMON   AND   M A  LICHTMAN ******
100     PRINT "   ******                                      ******
110     PRINT "   ******   UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE   ******
120     PRINT "   ******          ROCHESTER, NEW YORK 14642             ******
130     PRINT "   ******                                      ******
140     PRINT "   **************************************************
150     PRINT. PRINT: PRINT. PRINT· PRINT
160     PRINT "PRESS ANY KEY TO CONTINUE"
170     A$=INKEY$  IF A$=" " THEN 170
180     CLS
190     FOR M=0 TO 10: READ S(M, 1): NEXT M
200     FOR M=0 TO 10. READ S(M, 2): NEXT M
210     FOR M=0 TO 10: READ S(M, 3)· NEXT M
220     PRINT "SURVIVAL PREDICTION FOR CML PATIENTS TREATED WITH BONE MARROW TRANSPLANT"
230     PRINT
240     PRINT
250     PRINT
260     INPUT "ENTER PATIENT'S AGE (YEARS) "; AGE
270     IF AGE >50 GOTO 2030
280     CLS: PRINT
290     INPUT "ENTER TOTAL MEAN LIFE SPAN (NORMAL=70) (YEARS) "; DAGE
300     PRINT
310     T=DAGE-AGE: PRINT· PRINT· CLS
320     PRINT
330     INPUT "PROJECTED SURVIVAL VIA TRANSPLANTATION (%) "; K
340     SURV=K
350     K=K/100
360     INPUT "PROJECTED SALVAGE VIA TRANSPLANTATION IN ACCELERATED/BLASTIC PHASE (%) "; L
370     SALV-L
380     CLS
```

APPENDIX (Continued)

```
390     L=L/100
400     INPUT "DO YOU WANT TO ENTER PROGNOSTIC GROUP OR PATIENT PARAMETERS?
        (PG FOR PROGNOSTIC GROUP OR RETURN FOR PATIENT PARAMETERS)", W$
410     IF W$="PG" THEN 590
420     IF W$="pg" THEN 590
430     PRINT
440     PRINT "PARAMETERS TO DETERMINE PROGNOSTIC GROUP": PRINT: PRINT
450     INPUT "SPLEEN SIZE (CENTIMETERS BELOW LEFT COSTAL MARGIN) ";SPL: PRINT
460     INPUT "PLATELET COUNT (X 1000) "; PLT: PRINT
470     INPUT "PERCENTAGE OF BLASTS"; BLAST
480     A=EXP ((.0116)*(AGE-43.4))
490     B=((.0345)*(SPL-7.51))
500     C=((.188)*(((PLT/700)^2)-(.563)))
510     D=((8.869999E-02)*(BLAST-2.1))
520     FCT=A+B+C+D
530     IF FCT<.78 THEN P=1
540     IF FCT=.78 THEN P=2
550     IF (FCT>.78) AND (FCT<1.3) THEN P=2
560     IF FCT=1 3 THEN P=3
570     IF FCT>1 3 THEN P=3
580     CLS  GOTO 600
590     PRINT  INPUT "PROGNOSTIC GROUP= ";P
600     INPUT "FACTOR <1 FOR DECREASING TXP CURE WITH DELAY (ENTER 1.0 IF NO DECREASE) ";F
610     F=F^.5
620     INPUT "FILENAME FOR RESULTS ", D$: CLS
630     OPEN D$ FOR OUTPUT AS #1
640     PRINT#1, "OUTPUT FILE ";D$
650     PRINT "AGE= "; AGE
660     PRINT#1, "AGE= "; AGE
670     PRINT "MEAN LIFE SPAN= ", DAGE
680     PRINT#1, "MEAN LIFE SPAN= ", DAGE
690     IF FCT=0 GOTO 720
700     PRINT "RELATIVE RISK= ", FCT
710     PRINT#1, "RELATIVE RISK= "; FCT
720     PRINT "PROGNOSTIC GROUP= "; P
730     PRINT#1, "PROGNOSTIC GROUP= "; P
740     PRINT "SURVIVAL AFTER TRANSPLANT= ", K*100; "%"
750     PRINT#1, "SURVIVAL AFTER TXP= "; K*100; "%"
760     PRINT "SALVAGE AFTER TRANSPLANT= "; L*100; "%"
770     PRINT#1, "SALVAGE AFTER TXP= "; L*100; "%"
780     PRINT "FACTOR FOR DECREASING TXP CURE WITH DELAY "; F^2;" PER YR"
790     PRINT#1, "FACTOR FOR DECREASING TXP CURE WITH DELAY "; F^2," PER YR"
800     PRINT "       "
810     GRAL=0
820     PRINT "TXP TIME       ","CALC LIFE EXP";"        CLE/NLE       ";"CHANCE OF SURV"
830     PRINT#1, "TXP TIME      ";"CALC LIFE EXP";"        CLE/NLE       ";"CHANCE OF SURV"
840     PRINT
850     SAL=0
860     FOR T1=0 TO 5 STEP  5. REM TIME OF TRANSPLANT
870     J=T1/ 5
880     IF J=0 THEN 900
890     GRAL=GRAL+(S(J,P)+S((J-1),P))*.25
900     MYEXP=(1-L)*GRAL+L*T+(T-T1)*S(J,P)*(K-L)
910     TN=70-AGE
920     R=(MYEXP/TN)*100
930     C=((K*(S(J,P)))+(L*(1-(S(J,P)))))*100
940     IF MYEXP<T1 THEN R=0
950     IF MYEXP<T1 THEN GOTO 990
960     PRINT T1,         MYEXP.        R;"%",    C,"%"
970     PRINT#1, T1,         MYEXP.   R,          C
980     GOTO 1010
990     PRINT T1,         MYEXP,    ,             C;"%"
1000      PRINT#1,              MYEXP,              R,       C
1010
```

APPENDIX (Continued)

```
1020    K=F*K
1030    IF K<0 THEN K=0
1040    '
1050    L=F*L
1060    IF L<0 THEN L=0
1070    NEXT T1
1080    PRINT
1090    PRINT "TO CONTINUE TOUCH ANY KEY"
1100    A$=INKEY$:   IFA$="" THEN 1100
1110    CLOSE#1
1120    CLS:   CLOSE ALL
1130    DEFSNG A-Z
1140    OPEN D$ FOR INPUT AS #1
1150    COUNT=0
1160    SCREEN 2,0   : KEY OFF
1170    CLS
1180    DEF FNX(X) = 60 +X*550/XMAX
1190    DEF FNY(Y) = 16+(YMAX-Y)*160/YMAX
1200    GOSUB 1300      'INPUT DATA -------------------
1210    GOSUB 1370      'ESTABLISH X&Y AXES ----------
1220    GOSUB 1400      'DRAW X&Y AXES---------------
1230    GOSUB 1690      'DRAW DATA POINTS------------
1240    A$=INKEY$: IF A$="" THEN 1240
1250    CLOSE #1
1260    SCREEN 0,0,0: COLOR 7,1,1,: CLS: KEY OFF
1270    INPUT "DO YOU WANT TO ENTER MORE DATA? (Y OR N) "; AN$
1280    IF (AN$="Y") OR (AN$="y") THEN CLS: RUN
1290    END
1300    INPUT #1,A$       'INPUT DATA--------------
1310    IF (INSTR(A$, "TIME"))=0 GOTO 1300
1320    COUNT=COUNT+1
1330    IF EOF (1) THEN 1360
1340    INPUT #1, TXP (COUNT), CALC(COUNT), CLE(COUNT),CHANCE(COUNT)
1350    GOTO 1320
1360    RETURN
1370    XMAX - 10       'ESTABLISH X&Y AXES----------
1380    YMAX - 100
1390    RETURN
1400    'DRAW LABELS AND AXES---------------------
1410    LINE (FNX(0),FNY(YMAX))-(FNX(0),FNY(0))
1420    LINE -(FNX(XMAX),FNY(0))
1430    LOCATE 3,10: PRINT "--------->"
1440    LOCATE 3,21   PRINT "CLE/NLE IF NO DISEASE"
1450    LOCATE 3,1 : PRINT YMAX;
1460    LOCATE 7,2 . PRINT "PER-";
1470    LOCATE 8,2 : PRINT "CENT";
1480    LOCATE 13,1 : PRINT YMAX/2;
1490    LOCATE 24,41 . PRINT XMAX/2;
1500    LOCATE 24,75 : PRINT XMAX;
1510    LOCATE 25,36 : PRINT "YEARS AFTER DX";
1520    LOCATE 1,36   : PRINT D$
1530    LOCATE 5,42   : PRINT "CLOSED SYMBOLS: CLE/NLE"
1540    LOCATE 6,42   : PRINT "OPEN SYMBOLS· CHANCE OF SURVIVAL"
1550    LOCATE 10,46 : PRINT "AGE- "; AGE
1560    LOCATE 11,46 : PRINT "MEAN LIFE SPAN= ", DAGE
1570    IF FCT=0 THEN 1590
1580    LOCATE 12,46. PRINT "RELATIVE RISK= ", FCT
1590    LOCATE 13,46· PRINT "PROGNOSTIC GROUP= ", P
1600    LOCATE 14,46  PRINT "SURVIVAL AFTER TXP= "; SURV; "%"
1610    LOCATE 15,46: PRINT "SALVAGE AFTER TXP= ", SALV; "%"
1620    LOCATE 16,46  PRINT "DELAY FACTOR= "; F^2
1630    FOR Y-0 TO YMAX STEP YMAX/10
1640    LINE (58,FNY(Y))-(62,FNY(Y))
1650    NEXT Y
```

APPENDIX (Continued)

```
1660    FOR X=0 TO XMAX STEP XMAX/10
1670    LINE (FNX(X),174)-(FNX(X),178)
1680    NEXT X : RETURN '
1690    COUNT=COUNT-1
1700    FOR I=1 TO COUNT      'DRAWS SQUARES----------
1710    X=TXP(1)
1720    Y=CLE(1)
1730    IF CLE(1)=0   THEN GOTO 1750
1740    LINE (FNX(X)-2,FNY(Y)-2)-STEP(4,4),1,BF
1750    NEXT I
1760    I=0
1770    FOR I=2 TO COUNT      'DRAWS LINE----------
1780    X1 = TXP(I)
1790    X2 = TXP(I-1)
1800    Y1 = CLE(I)
1810    Y2 = CLE(I-1)
1820     IF Y1=0 GOTO 1840
1830     LINE    (FNX(X1),FNY(Y1))-(FNX(X2),FNY(Y2))
1840    NEXT   I
1850    I=0
1860    FOR  I=1 TO COUNT
1870    X=TXP(I)
1880    Y=CHANCE (I)
1890    LINE    (FNX(X)-2,FNY(Y)-2)-STEP(4,4),,B
1900    NEXT I
1910    FOR I=2 TO COUNT      'DRAWS LINE----------
1920    X1 = TXP(I)
1930    X2 = TXP(I-1)
1940    Y1 = CHANCE(I)
1950    Y2 = CHANCE(I-1)
1960    LINE (FNX(X1),FNY(Y1))-(FNX(X2),FNY(Y2))
1970    NEXT I
1980    RETURN
1990    '
2000    DATA 1,.99,.97,.93,.88,.84,.78,.72,.66,.61,.55
2010    DATA 1,.98,.95, 88, 80, 72, 64, 54, 47,.42,.38
2020    DATA 1,.97,.87,.74, 63, 50,.39, 29,.21,.16,.12
2030    CLS: PRINT "PATIENT NOT ELIGIBLE FOR TRANSPLANT IF AGE >50 YEARS !": END
```

The authors will provide a copy of this program to interested readers who supply a blank 5¼" magnetic disk that will run on IBM-compatible computers.

Malignant Clonal Expansion of Large Granular Lymphocytes With a Leu-11+, Leu-7− Surface Phenotype: In Vitro Responsiveness of Malignant Cells to Recombinant Human Interleukin 2

Shoichi Koizumi, Hidetoshi Seki, Tomoko Tachinami, Masashi Taniguchi, Akira Matsuda, Kazuyuki Taga,
Takayuki Nakarai, Eiji Kato, Noboru Taniguchi, and Hideo Nakamura

A 14-year-old Japanese female with neutropenia showed malignant proliferation of the large granular lymphocytes (LGLs). These LGLs were E rosette+ and Fc(IgG) receptor+ and therefore are referred to as Tγ lymphocytes. They were also Leu-11+ and OKT11+; however, they were clearly negative for Leu-7, OKT3, OKT8, OKM1, and HNK-1 antigens as well as for terminal deoxynucleotidyl transferase activity. Karyotype analysis revealed 47, XXX. The LGLs showed no rearrangement of T cell receptor Cβ genes. The natural killer (NK) cell activity against K562 target cells was low, but was significantly augmented after stimulation by recombinant human interleukin 2 (IL 2) in contrast to minimal NK boosting by recombinant human γ-interferon (γ-IFN). Such a unique responsive ability to lymphokines was quite similar to that noted in fetal and cord blood cells. These LGLs also demonstrated a considerable increase in antibody-dependent cell-mediated cytotoxicity (ADCC) and lymphokine-activated killer (LAK) activity after a short incubation with IL 2. Although in a resting stage they showed no IL 2 receptor expression as examined by anti-Tac antibody, Tac antigen appeared after IL 2 treatment followed by a marked increase in ^{3}H-thymidine incorporation and a remarkable production of γ-IFN. To investigate the mechanism of neutropenia, in vitro IL 2–stimulated coculture studies of these cells with normal bone marrow cells were performed. Colony formation of myeloid progenitors (CFU-C) was significantly suppressed. In addition, the conditioned medium from IL 2–stimulated LGLs indicated a remarkable suppression of CFU-C. These results suggest that these LGLs with a Leu-11+, Leu-7− surface phenotype might belong to a unique subset of pre-NK cells that are functionally and phenotypically similar to those represented at any early stage of human ontogeny and that they strongly express Tac antigen under the influence of IL 2 administration, followed by remarkable cell proliferation and γ-IFN production.

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NATURAL KILLER (NK) cells, morphologically indistinguishable from most large granular lymphocytes (LGLs), play a role in the immunologic system[1-3] as well as in the regulation of hematopoiesis.[4] Because these cells express receptors for both sheep red blood cells (SRBCs) and the Fc portion of IgG, they are referred to as Tγ lymphocytes.[5] Although the cellular lineage of the LGLs still remains to be identified, recent studies using a panel of monoclonal antibodies including Leu-7 and Leu-11 have revealed several different subsets having different NK cell activities.[6,7] In addition, on the basis of the responsive ability to recombinant human lymphokines such as γ-interferon (γ-IFN) and interleukin 2 (IL 2), different populations of so-called human pre-NK cells in fetal and cord blood cells were demonstrated.[8,9]

A number of patients with abnormal proliferation of LGLs and chronic neutropenia have been described in the last several years.[10-36] The majority of cases showed pan-T cell (OKT3) and cytotoxic/suppressor T cell (OKT8) phenotypes as well as Leu-7 and Leu-11, which are associated with NK cell phenotypes. Because these cases usually presented rather chronic clinical courses, they were diagnosed as having either T cell chronic lymphocytic leukemia (T-CLL)[10,18,19,25-27,33,35] or Tγ lymphocytosis[5,13,17,21,23] rather than a primary neoplastic process.

In contrast to the cases previously reported, our case showed a leukemic expansion of LGLs, which were found to be associated with a considerable NK cell activity and have a unique Leu-11+, Leu-7− phenotype. This study identifies the immunologic and hematologic characteristics of these proliferative cells and defines their origin during the ontogenic development of NK cells. The possible suppressive effect of these LGLs on normal hematopoietic progenitor cells is also discussed.

CASE REPORT

On Aug 19, 1985, a 14-year-old Japanese female was admitted to the Japanese Red Cross Hospital in Kanazawa because of a high fever that had lasted ten days. She was physically well developed for her age. Family history was noncontributory. The examination revealed hepatomegaly 4 cm below the right costal margin and splenomegaly 1 cm below the left costal margin. Laboratory results disclosed the following values: hemoglobin (Hb), 10.0 g/dL; RBC, 3,500,000/μL; and WBC, 11,400/μL with 1% granulocytes and 99% lymphocytes. The platelet count was 90,000/μL. Serum chemistry levels were as follows: SGOT, 95 IU/L; SGPT, 46 IU/L; and lactic dehydrogenase (LDH), 439 IU/L. During her hospitalization, the high fever continued despite antibiotic treatment and a moderate dose of prednisone.

On Sept 4, the patient was transferred to the Kanazawa University Hospital for assessment and treatment. At that time, hepatomegaly measured 4.5 cm and splenomegaly 9.5 cm. No significant lymph node swelling was observed. A complete blood count showed the following values: Hb, 11.5 g/dL, RBC, 4,270,000/μL; and WBC

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14,300/μL with 5% granulocytes and 95% lymphocytes. The platelet count was 119,000/μL. A May-Grünwald-Giemsa-(MGG)-stained peripheral blood smear demonstrated a predominance of large lymphocytes (84%) 15 to 20 μm in diameter, with abundant cytoplasm and sparse intracytoplasmic granules (Fig 1A). Bone marrow aspiration also revealed increased numbers of abnormal large granular lymphocytes (LGLs) with 42% nucleated cells. Blood chemistries showed the following data: total bilirubin, 0.60 mg/dL; SGOT, 71 IU/L; SGPT, 28 IU/L; LDH, 695 IU/L; γ-glutamil transpeptidase (γ-GTP), 195 IU/L; BUN, 14 mg/dL; creatinine, 0.9 mg/dL; and uric acid, 4.5 mg/dL. Albumin and cholinesterase levels were normal. Tests for rheumatoid arthritis and lupus erythematosus as well as the Coombs' test were all negative. Polyclonal hypergammaglobulinemia was demonstrated (IgG, 2,082 mg/dL; IgA, 626 mg/dL; and IgM 267 mg/dL). The C3c and C4 levels were 121 mg/dL and 69 mg/dL, respectively. Antibodies to Epstein-Barr virus (EBV) as detected by indirect immunofluorescence techniques showed the following titers: EBV capsid antigen (VCA) IgG, 320; IgM, 10; early antigen (EA), 80; and EB nuclear antigen (EBNA), 160. The titer of antibody to cytomegalovirus was within the normal range. The tests for hepatitis B surface antigen (HBsAg) and anti-HBsAg antibodies were negative. Although treatment with prednisone (60 mg/d) continued and a pulse therapy of methyl prednisone (1,000 mg/d × 3) was also given after informed consent, the high fever persisted. The WBC count gradually increased to 126,000/μL (LGLs, 80%) at the end of September. The LDH level also increased to 16,216 IU/L. A second bone marrow aspiration after informed consent showed a further increase in the numbers of abnormal LGLs (79%). Blood levels of γ-IFN were checked twice in the clinical course by radioimmunoassay.[37] The levels were 27.2 U/mL on Sept 6 and 5.3 U/mL on October 1, both of which were markedly increased as compared with normal controls (less than 0.2 U/mL). However, the presence of IL 2 in plasma could not be identified by a sensitive radioimmunoassay method using a monoclonal antibody against human IL 2, L61 (kindly performed by Dr Ide, Shionogi & Co, Ltd, Osaka, Japan). At the beginning of Oct, a remarkable swelling of the bilateral cervical lymph nodes was noted. Full doses of anticancer drugs (vincristine, doxorubicin, cyclophosphamide, and cytosine arabinoside) were started, but the WBC count further increased to 200,000/μL (LGLs, 90%). Chromosome analysis of the LGLs using a G-banding technique revealed an abnormal 47, XXX pattern. When Southern blot techniques were used, the LGLs were not found to rearrange the T cell receptor Cβ genes. The patient died on Oct 12 of a hemorrhagic tendency caused by disseminated intravascular coagulation. Autopsy was refused.

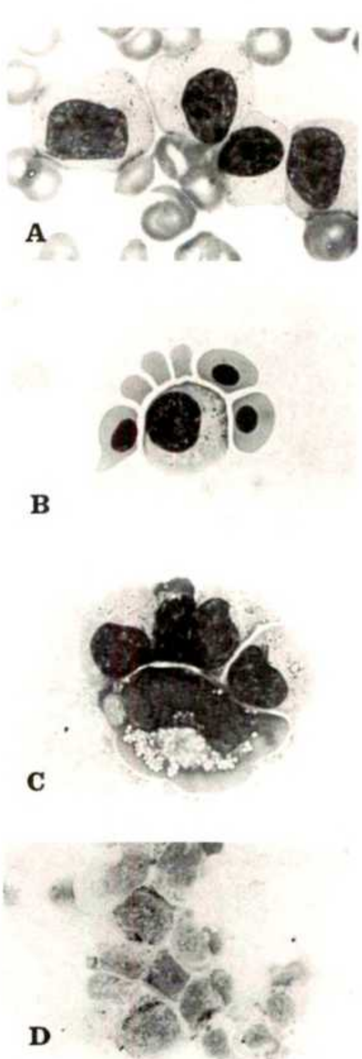

Fig 1. Morphologic characteristics of the proliferative cells. A. Peripheral blood smear illustrating four cells similar to LGLs (MGG stain; original magnification × 1,000; current magnification × 400). B. LGL double rosetted with sheep RBCs and nucleated chicken RBCs coated with IgG (MGG stain; original magnification × 1,000; current magnification × 400). C. LGL bound with a K562 cell after 18-hour incubation of NK activity assay (MGG stain; original magnification × 1,000; current magnification × 400). D. Expression of intracellular γ-IFN produced by the stimulation of IL 2 (Immunoalkaline phosphatase stain; original magnification × 400; current magnification × 160).

MATERIALS AND METHODS

Surface markers. Peripheral blood mononuclear cells (PBMCs) from the patient and normal donors who had given informed consent were isolated by Lymphoprep (Nyegaard and Co, Oslo) gradient centrifugation as described elsewhere.[38] The expression of receptors for SRBCs (ER) and Fc receptors for IgG (Fc(IgG)R) were determined by the rosetting method using either SRBCs or chicken RBCs sensitized with rabbit IgG antibody against chicken RBCs.[39,40] PBMCs were studied with a panel of monoclonal antibodies: OKT3 (Ortho Diagnostic Systems, Raritan, NJ), which reacts with mature T cells; OKT11 (Ortho), which binds to SRBC receptors; OKT4 and OKT8 (Ortho), which selectively bind to lymphocyte subsets containing cells with helper and suppressor/cytotoxic activity, respectively; OKM1 (Ortho), which identifies an antigen expressed on cells from the myelomonocytic lineage and some LGLs; OKT10 (Ortho), which reacts with the majority of thymocytes and activated T cells and has been reported to recognize some LGLs; Anti-HLA-DR (Becton Dickinson Monoclonal Center, Inc, Mountain View, Calif), which reacts with Ia-like antigens; Leu-7 (Becton Dickinson), which binds to LGLs and about 20% of PBMCs; NKH-1 (Coulter Immunology, Hialeah, Fla), which identifies NK cells but not neutrophils; and Leu-11 (Becton Dickinson), which reacts with the Fc(IgG)R of NK cells and neutrophils. We also used anti-Tac antibody (provided by Dr Waldmann, National Cancer Institute, Bethesda, Md), which recognizes an IL 2 receptor-related antigen.[41] Monoclonal antibodies were used in a direct or an indirect immunofluorescence technique, and the surface markers were analyzed by using a fluorescence-activated cell analyzer (Ortho Spectrum III, Ortho Diagnostics, Westwood, Mass).

Recombinant human IL 2. IL 2 was a product of S.A. Biogen, Geneva. This IL 2 preparation had a titer of 22×10^3 U/mL with a specific activity of 1.15×10^6 U/mg of protein (one unit being the amount of IL 2 giving 50% of the maximal ^{3}H-thymidine [^{3}H-TdR] incorporation into the NK-7 clone assay as described by Suzuki et al[42]). One such unit is equivalent to 4.5 units of a reference Jurkat IL 2 standard provided by the Biological Response Modifiers Program of the National Cancer Institute.

Response to lymphokines. Proliferative responses of PBMCs from the patient and normal donors to phytohemagglutinin (PHA-P, 0.1%, Difco, Laboratories, Detroit) and recombinant IL 2 (25 U/mL) were assessed in triplicate by ^{3}H-TdR incorporation as previously described.[9,39]

Cytotoxic activity. NK cell activity was measured in a four-hour ^{51}Cr-release assay using K562 target cells as previously described.[8] In brief, various concentrations of effector cells were suspended in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 300 μg/mL L-glutamine, 200 U/mL penicillin, 10 μg/mL gentamicin, 25 mmol/L HEPES buffer (GIBCO), and 5% fetal bovine serum (FBS, M.A. Bioproducts, Walkersville, Md). In some experiments, to study the role of γ-IFN (provided by Shionogi & Co, Ltd) and IL 2 in the augmentation of NK cell activity, cells were pretreated with 25 U/mL IL 2 or 1,000 U/mL γ-IFN for 18 hours prior to assay. Cells were added to triplicate cultures of 10^4, ^{51}Cr-labeled K562 cells in 0.2 mL vol in V-bottomed microtiter plates (Nunk, Kamstrup, Denmark). After centrifugation at 25 g for eight minutes, the cells were incubated for four hours at 37 °C in a humidified 5% CO_2 atmosphere. The supernatants were harvested with a Titertek supernatant collection system (Flow laboratories, Walkersville, Md), and radioactivity was determined with a gamma counter. The cytotoxicity was calculated as follows: percent cytotoxicity = (experimental release − spontaneous release) × 100/(total release − spontaneous release).

The lymphokine-activated killer (LAK) activity was assayed by the aforementioned method using Hela, Daudi, BALL, and EL4 cells as NK-resistant tumor cell lines after the effector cells were pretreated with or without IL 2. The assay for antibody-dependent cell-mediated cytotoxicity (ADCC) was performed by a ^{51}Cr-release method using chicken RBCs coated with rabbit IgG against chicken RBCs. PBMCs from normal donors, cord blood mononuclear cells, and Leu-11+ cell populations sorted by a cell sorter (Epics C, Coulter Electronics, Hialeah, Fla) as described[9] were used as controls.

γ-IFN assay. γ-IFN activity in the PHA-P– or IL 2–induced LGL supernatants was assayed by the capacity to inhibit the cytopathic effect of Sindbis virus on FL monolayers as described by Langford et al.[43] All twofold titrations were carried out in duplicate, and the antiviral activity was expressed as the reciprocal of the highest sample dilution that reduced the viral cytopathic effect by 50%. To determine whether antiviral activity in the supernatants belonged to the γ type, heat and pH stability tests were performed as previously described.[44]

Intracellular expression of γ-IFN and IL 2 were identified by a routine immunohistochemical technique[45] using a monoclonal anti–γ-IFN antibody (B3, kindly provided by Dr Le, New York University Medical Center, NY) and a monoclonal antihuman IL 2 antibody, L61 (kindly provided by Dr Ide, Shionogi & Co, Ltd), respectively, on acetone-fixed cytocentrifuge preparations.

CFU-C assay. To investigate the effect of PBMCs from the patient on myeloid progenitor cells in bone marrow, in vitro coculture studies of PBMCs with normal bone marrow cells were performed. Normal bone marrow mononuclear cells (BMMCs) were obtained from volunteers after informed consent and were separated by gradient centrifugation as described.[46] CFU-C assays were performed by a modified semisolid agar method[47] originally described by Pike and Robinson.[48] BMMCs (10^5/mL) and PBMCs (1 to 2×10^5/mL) were mixed and preincubated in Iscove's modification of Dulbecco's medium (IMDM, Flow) containing 300 μg/mL L-glutamine, 200 U/mL penicillin, 10 μg/mL gentamicin, and 10% FBS for 18 hours at 37 °C. After incubation the cell mixture was plated into 0.35% agar (Bacto-agar, Difco) in IMDM containing 20% FBS with or without IL 2 (25 U/mL). Mo-CM (kindly provided by Dr Golde, UCLA, Los Angeles) was used throughout this study as a source of colony-stimulating factor at a concentration of 10%. After 14 days of incubation at 37 °C in a humidified 5% CO_2 atmosphere, colonies of 40 cells or more were counted.

Culture supernatants of IL 2–stimulated PBMCs. An aliquot of the patient's PBMCs (2×10^6/mL) was cultured in RPMI 1640 medium containing 10% FBS and with or without IL 2 (25 U/mL). After incubation for 48 hours at 37 °C, the cells were pelleted by centrifugation, and the supernatants were harvested and stored at −20 °C until used. To examine the effects of the supernatants on CFU-C, graded amounts of the supernatants were added to the CFU-C assay system just described. To determine the category of IFN to which the activity of the supernatants was attributable, we performed a neutralization study using an anti–γ-IFN antibody and a specific antiserum to human α-IFN (Interferon Sciences, Inc, New Brunswick, NJ). IL 2–stimulated cell supernatants were treated with an excess of each antibody at 37 °C for one hour prior to CFU-C assay.

RESULTS

Morphologic characteristics. MGG-stained smears showed that the morphologic characteristics of the proliferative cells from this patient were quite similar to LGLs. They looked relatively large (15 to 20 μm in diameter), with a high cytoplasm-to-nucleus ratio and sparse azurophilic granules in the cytoplasm (Fig 1A). Nuclei were round, and nucleoli were rarely observed. Double-nucleated cells were noted rarely. An electron microscopic analysis revealed prominent cytoplasmic granulogenesis and cytoplasmic inclusion bodies. Parallel tubular arrays that were characteristic of normal human Tγ lymphocytes were also indicated. Cytochemical analysis revealed that they were all negative for esterase, peroxidase, acid phosphatase, terminal deoxynucleotidyl transferase, and asialo GM1 activities.

Surface markers. Immunologic analysis using a panel of monoclonal antibodies showed that these LGLs had receptors for SRBCs and Fc receptors for IgG that were identified by rosetting with both SRBCs and chicken RBCs (Fig 1B). In addition, these cells had only a OKT11 antigen among a series of T cell–related antigens, OKT11 and Leu-11 antigens as NK cell–related antigens, and OKT10 and Ia-like antigens as activated T cell–related antigens. However, of interest, there was no expression of Leu-7, OKT3, OKT8, or OKM1 antigen on the surface of the LGLs (Fig 2A and B).

NK cell activity. Figure 3 shows NK cell activities in the patient's LGLs against K562 target cells as compared with those in sorted Leu-11+ cells from adult PBMCs and cord blood cells. The spontaneous NK cell activity of the patient's LGLs was much lower than that in the adult control, but was significantly augmented after treatment with IL 2 to almost the same level as that in adult Leu-11+ cells at various effector/target cell ratios. In contrast, after stimulation by γ-IFN, no marked increase of the NK activity was demonstrated. Such a characteristic ability of different responsiveness to these lymphokines was quite similar to that shown in the control cord blood Leu-11+ cells. The LGLs were tested

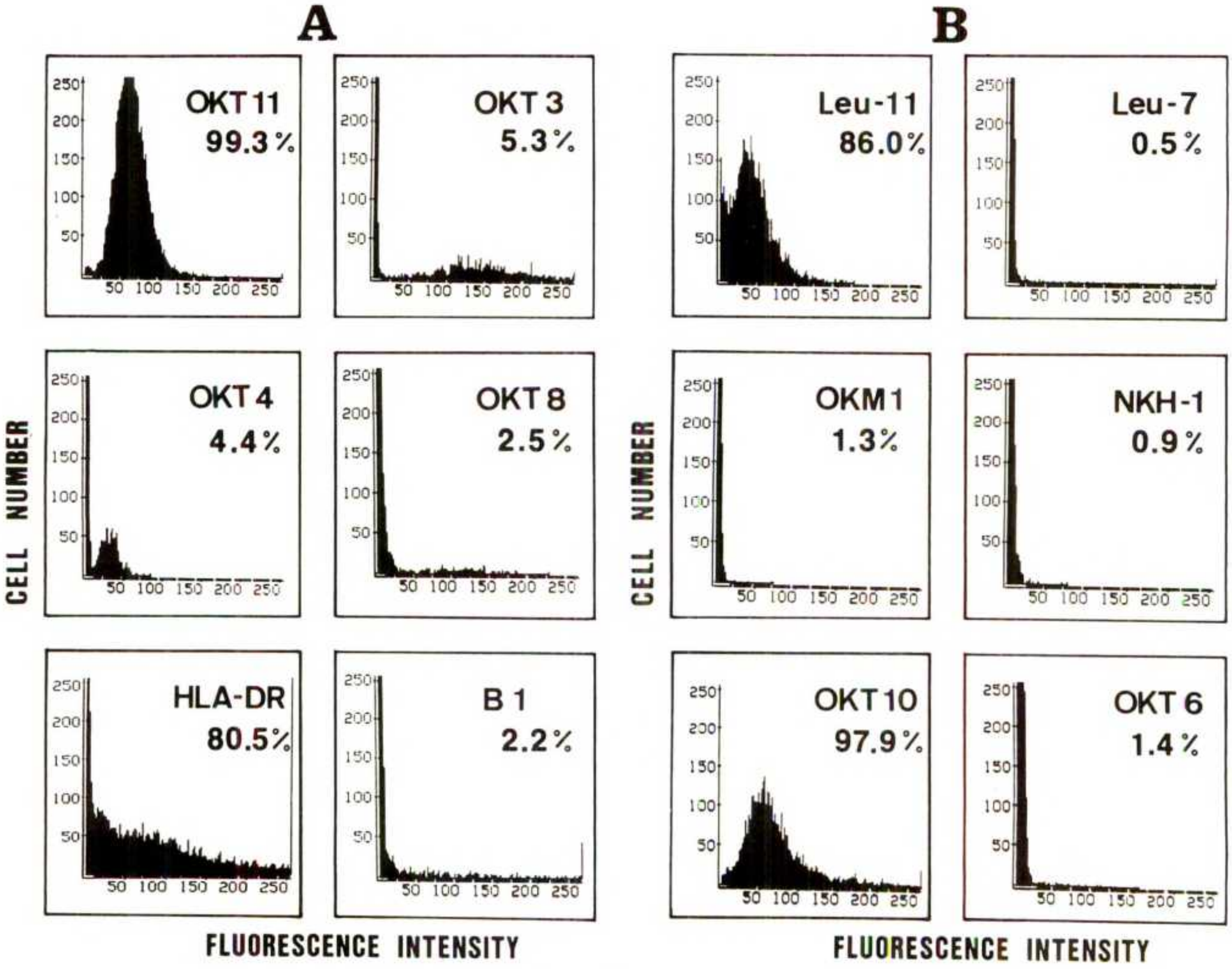

Fig 2. (A, B) Surface markers of the patient's LGLs.

for their ability to bind and kill K562 target cells in a single-cell cytotoxicity assay in agarose (Fig 1C). About 25% of the patient's LGLs had a binding capability to K562 target cells, and the relative proportion of target-bound cells was increased to approximately 43% after treatment with IL 2. The proportion of dead cells among these conjugates was also increased from 28% of the patient's LGLs to 75% after IL 2 stimulation.

LAK activity. LAK activity of these LGLs was assessed by using four different kinds of cell lines as NK-resistant tumor cells as shown in Fig 4. A remarkable enhancement of IL 2–induced LAK activities was exhibited at various effector/target ratios, suggesting that these LGLs have a wide spectrum of target specificity.

ADCC. ADCC of the patient's LGLs was compared with that of normal adult PBMCs at different effector/target ratios. The patient's LGLs had approximately two- to threefold higher levels of ADCC (80%) than the control adult PBMCs (30%) at an effector/target ratio of 20/1.

Response to lymphokines and IFN production. Proliferative response of the patient's LGLs to PHA-P and IL 2 was measured by ^{3}H-TdR incorporation. As compared with the control adult PBMCs, the patient's LGLs showed a remarkable increase of ^{3}H-TdR uptake by the stimulation of IL 2, whereas PHA-P was less effective on the patient's LGLs than the adult PBMCs (Table 1). In addition, a significant increase of IFN production was demonstrated, especially by the influence of IL 2 administration (Table 2). Characterization of IFN produced by the LGLs was examined by heat and pH stability tests. A marked reduction of IFN titers after the treatment with both hot water (56 °C) and acid solution (pH 2) was shown, suggesting that the γ type of IFN was contained in the supernatants of the PHA-P– and IL 2–stimulated cultured media. In concordance with the increase of γ-IFN activity in the supernatants, intracellular γ-IFN produced by IL 2 was clearly demonstrated by an immunohistochemical technique using a monoclonal anti–γ-IFN antibody (Fig 1D). IL 2–containing cells were not identified immunohistochemically even after exogenous IL 2 stimulation.

Tac antigen expression. Figure 5 shows the ability of Tac antigen expression on the patient's LGLs after IL 2 stimulation. They expressed marginal amounts of Tac antigen on their surfaces in a resting stage, and the proportion of

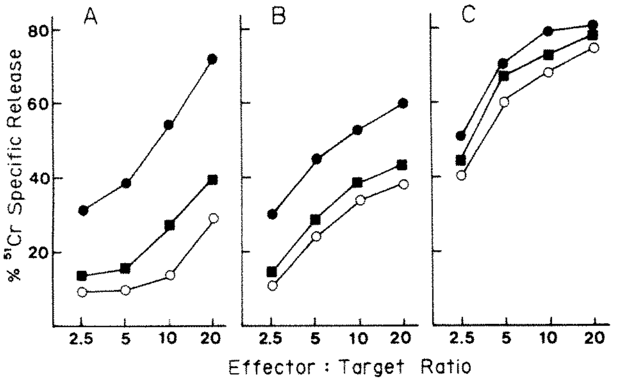

Fig 3. NK cell activity of the patient's LGLs and augmentation by IL 2. The patient's LGLs (A) and sorted Leu-11+ cells from cord blood (B) and a normal adult donor (C) were incubated with K562 target cells at different effector/target ratios after stimulation with or without IL 2 and γ-IFN as described in Materials and Methods. (Key: ○—○, without stimulations; ●—●, with IL 2 stimulation; ■—■, with γ IFN stimulation)

Tac+ cells was not increased by PHA-P stimulation. However, when these LGLs were cultured with IL 2, they exhibited a rapid (even within 12 hours of incubation) and significant augmentation of the expression of Tac antigen in contrast to control adult PBMCs. γ-IFN induced no significant increase in Tac antigen expression on the patient's LGLs.

CFU-C assay. Severe neutropenia was shown throughout the clinical course of this patient. Thus, we next studied suppressive effects of the patient's LGLs on in vitro colony formation of CFU-C. The patient's bone marrow (LGLs, 42%) showed a significantly decreased number of CFU-C ($29/10^5$ BMMCs) as compared with normal controls (131 ± 46 [mean ± SD] $/10^5$ BMMCs). No suppressive effect of LGLs on normal bone marrow CFU-C formation without stimulation of IL 2 was shown, whereas a remarkable suppression of CFU-C was demonstrated under the influence of IL 2 administration (Table 3). Furthermore, the IL 2-induced cell supernatants exhibited a dose-dependent suppressive effect on CFU-C formation (Table 4). As shown in Table 5, the suppressive activity of the supernatants was markedly abolished with anti-γ-IFN, but not anti-α-IFN, antibody treatment, suggesting that γ-IFN could be greatly involved in the suppression of granulopoiesis.

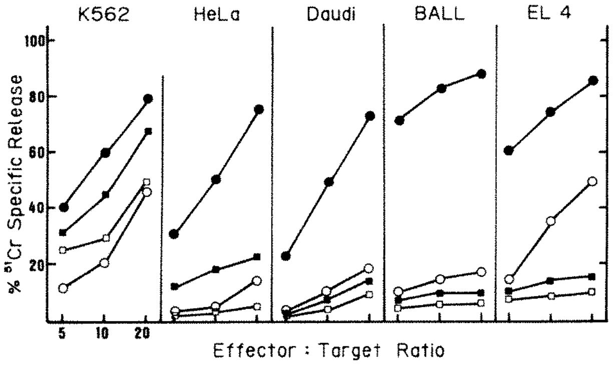

Fig 4. LAK activity of the patient's LGLs as compared with that of PBMCs from a normal donor. Cells were cultured with four different kinds of ^{51}Cr-labeled cells (Hela, Daudi, BALL, and EL4) as NK-resistant target cell lines after stimulation with or without IL 2. Key: ○—○, the patient's LGLs without IL 2; ●—●, the patient's LGLs with IL 2; □—□, PBMCs without IL 2; ■—■, PMBCs with IL 2.

Table 1. Proliferative Response of Patient's LGLs

	^{3}H-TdR Incorporation (cpm)		
	Medium	PHA-P	IL 2
Patient's LGLs	172 ± 23	1,468 ± 170	27,072 ± 2,838
Control PBMCs	210 ± 68	60,983 ± 1,988	2,013 ± 430

The patient's LGLs and control PBMCs from a normal adult donor were incubated with PHA-P (0.1%) for three days or IL 2 (25 U/mL) for five days. At 12 hours before harvest, 0.2 μCi ^{3}H-TdR was added, and the uptake of ^{3}H-TdR was measured. The data are expressed as mean ± SD for triplicate cultures.

DISCUSSION

In the present study, we described a patient with abnormal clonal expansion of LGLs and neutropenia. As these proliferative cells demonstrated ER+ and Fc(IgG)R+ phenotypes, they were referred to as Tγ lymphocytes. Recently, a number of cases of abnormal proliferation of Tγ lymphocytes (Tγ lymphoproliferative diseases, Tγ-LPD) have been reported[10-36] (for review see refs 5, 32, and 35), and most cases were not aggressive, although the majority of the patients had recurrent bacterial infections requiring antibiotic therapy. In contrast, our patients showed a very rapid expansion of Tγ lymphocytes. The patient's Tγ lymphocytes were similar to normal LGLs although they were much larger in size (more than 15 μm in diameter) and scanty in granules. They were positive for OKT11 and Leu-11 antigens. However, interestingly, they were negative for Leu-7, OKT3, and OKT8 antigens, which were expressed in most of the previously reported cases with Tγ-LPD. Such unique morphologic and phenotypic properties allows us to consider the lineage of these LGLs from which they abnormally expanded. Abo et al[7] recently characterized three subpopulations of human LGLs from blood and lymphoid tissues using HNK-1 (Leu-7) and Leu-11 monoclonal antibodies. Each subpopulation (HNK-1+ Leu-11−, HNK-1+ Leu-11+, and HNK-1− Leu-11+) had a different NK cell activity. Among these three subsets, the HNK-1+ subset lacking the Leu-11 appeared to be the most immature form of LGLs and had minimal NK cell activity, whereas the HNK-1− Leu-11+

Table 2. Characterization of IFN Produced by the Patient's LGLs

	IFN titers (U/mL) After Treatment With		
IFN Inducers	None	56 °C	pH 2
None	2	ND	ND
PHA-P (0.1%)	256	2	16
IL 2 (25 U/mL)	1,024	16	2

The supernatants of cultured-conditioned medium of the patient's LGLs stimulated with PHA-P or IL 2 were collected after three and five days of incubation, respectively. Heat and pH stability tests were performed, and IFN titers were measured as described in Materials and Methods.

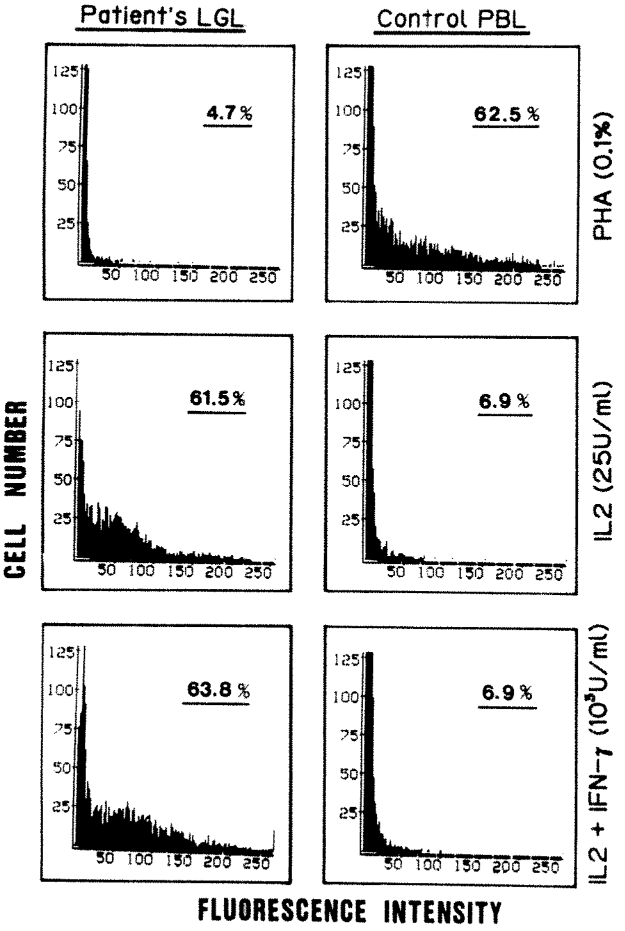

Fig 5. Tac expression of the patient's LGLs. The patient's LGLs and PBMCs from a normal donor as controls were incubated with PHA-P (0.1%), IL 2 (25 U/mL), or IL 2 plus γ-IFM (1,000 U/mL) for 24 hours. After incubation Tac+ cells were determined by an indirect immunofluorescence technique.

Table 3. Effects of the Patient's LGLs on CFU-C Growth

Bone Marrow Cells (/mL)	Effector Cells (/mL)	IL 2 (25 U/mL)	CFU-C (/10^5 Cells)	CFU-C Percentage
1 × 10^5	—	—	254	100
1 × 10^5	—	+	262	103
	Patient's LGLs			
1 × 10^5	1 × 10^5	—	262	103
1 × 10^5	2 × 10^5	—	324	128
1 × 10^5	1 × 10^5	+	57	22.4
1 × 10^5	2 × 10^5	+	43	16.9
	Control PBMCs			
1 × 10^5	1 × 10^5	—	327	129
1 × 10^5	2 × 10^5	—	335	132
1 × 10^5	1 × 10^5	+	254	100
1 × 10^5	2 × 10^5	+	274	108

The patient's LGLs were mixed with normal BMMCs and preincubated for 18 hours at 37 °C with or without IL 2 (25 U/mL). After incubation, the cell mixture was plated in 0.35% agar for the CFU-C assay. The number of colonies are expressed as mean values in triplicate cultures. Data from one of three similar experiments are presented.

subpopulation had a higher level of NK activity and was considered a more mature subset. Based on the expressions of Leu-11 and Leu-7 (HNK-1) antigens, the LGLs from our patient (Leu-11+, Leu-7−) seem to belong to a rather mature subset of NK cells. This speculation, however, is questionable because the patient's LGLs do not have sufficient spontaneous NK cell activity nor do they express OKT3 or OKT8 antigens, which have been reported to be expressed in mature NK cells.

Functional analysis of the responsiveness to IL 2 and γ-IFN is another useful method for studying the lineage of the LGLs. The NK cell activity of the patient's LGLs against K562 target cells was low but was significantly augmented by the stimulation of IL 2 in contrast to γ-IFN. In previous studies, Ueno et al[8] tested the ontogenic changes in the lymphokine responsiveness of human NK cells by using PBMCs from fetuses, premature infants, and full-term neonates as compared with adults. PBMCs from fetuses at early gestational ages showed minimal NK cell activity even after pretreatment with γ-IFN. In contrast, IL 2 induced a marked NK cell activity. On the other hand, PBMCs from full-term neonates showed a low level of NK cell activity but a considerable NK boosting by both γ-IFN and IL 2. From these data, ontogenic pre-NK cells from very early stages of human life were suggested to be divided into γ-IFN–sensitive and IL 2–sensitive subpopulations. Although it is not known whether these cell subsets belong to different cell lineages or different maturational stages of the same cell lineage, the patient's LGLs, which showed IL 2 but not γ-IFN sensitivity, might belong to a subset similar to fetal pre-NK cells. Furthermore, although Leu-7+ cells were negligible in cord blood, the relative proportion of Leu-11+ cells was shown to be equal to that in adult PBMCs by Seki et al.[9] They selected for a Leu-11+ cell population by a cell sorting technique and demonstrated that the spontaneous NK cell activity of Leu-11+ cells in cord blood was low but was significantly augmented under the influence of IL 2 administration. The abnormal LGLs from the patient, which have a similar phenotype to the NK cells in cord blood (Leu-11+ Leu-7−), also had a similar responsiveness to the lymphokines. This fact strongly suggests that the patient's LGLs may have originated from the pre-NK cell subset during maturational stages.

Table 4. Effects of IL 2–Stimulated LGL Supernatants on CFU-C Growth

Colony-Stimulating Factor (Mo-CM) Mixed With	CFU-C Per 10^5 Cells	CFU-C Percentage
None	502	100
Medium with IL 2 (10%)	565	112
Supernatant		
2.5%	167	33.3
5%	163	32.5
10%	129	25.7
15%	105	20.9

IL 2–stimulated LGL supernatants were collected after 48 hours of incubation at 37 °C. Graded amounts of the supernatants indicated were added to the CFU-C assay system using Mo-CM as a source of colony-stimulating factor as described in Materials and Methods. The numbers of colonies are expressed as mean values in triplicate cultures. Data from one of three similar experiments are presented.

Table 5. Influence of an Anti-γ-interferon (γ-IFN) Antibody on the Suppressive Effect of IL 2–Stimulated LGL Supernatants

Colony-Stimulating Factor (Mo-CM) Mixed With	CFU-C			
	Exp 1		Exp 2	
	Per 10^6 Cells	%	Per 10^6 Cells	%
None	448	100	157	100
Medium with IL 2 (5%)	422	94.2	170	108
5% supernatant	72	16.1	38	22 9
5% supernatant + anti–γ-IFN	293	65 4	128	81.5
Anti–γ-IFN	431	96.2	140	89.2
5% supernatant + anti–α-IFN	—	—	39	24 8
Anti–α-IFN	—	—	130	82 8

IL 2–stimulated supernatants were treated with an excess of antihuman γ-IFN or antihuman α-IFN antibody at 37 °C for one hour prior to CFU-C assay. The numbers of colonies are expressed as mean values in triplicate cultures

Pathologic clonal proliferation of the LGLs in this patient was also suggested by the karyotype analysis that showed an abnormal 47, XXX pattern. However, these LGLs were not found to rearrange the T cell receptor Cβ genes with Southern blot techniques (data not shown). The heterogeneity of Tγ-LPD in the organization of the Cβ genes has been shown by Rambaldi et al[49] who demonstrated that the more frequently encountered cases of T3+ Tγ-LPD showed clonal rearrangements (T3+ Cβ+ Tγ-LPD), whereas the less common T3– Tγ-LPD exhibit the germline Cβ configuration (T3– Cβ– Tγ-LPD). Our case (T3– Cβ–) confirms these findings.

Other unique functional capabilities caused by stimulation of the IL 2 of these LGLs were strong and rapid expression of Tac antigen followed by a marked cellular proliferation and a significantly increased production of γ-IFN. Although exogenous γ-IFN did not induce the expression of Tac antigen and there was no detectable IL 2 activity in cultured-conditioned medium or in blood, it is likely that the patient's LGLs do express Tac antigen and enhance their own abnormal growth via Tac antigen induction. A similar observation was made by Yodoi et al[50] who established a YT cell line derived from a boy with thymic lymphoma. These cells had NK cell surface phenotypes and NK-like killing activity. YT cells, which marginally expressed Tac antigen, markedly increased the expression of Tac antigen after incubation with IL 2–containing conditioned medium from peripheral blood leukocytes or spleen cells, both of which were stimulated with PHA.

This patient had severe granulocytopenia (less than 500/μL) as did approximately half (46%) of the patients with Tγ-LPD.[5] Some investigators suggested that Tγ cells and NK cells inhibited granulopoietic progenitor cells in vitro.[51,52] In this case a significant reduction of CFU-C formation was also noted when the patient's LGLs were added to a normal bone marrow CFU-C assay system. In addition, the inhibition of CFU-C was shown to be mediated by an IL 2–induced factor(s) (Table 4). A recent study by Degliantoni et al[53] suggested that the effect of NK cells on hematopoietic progenitors was mediated through a specific soluble factor(s) (NK-derived colony-inhibited activity; NK-CIA) that was released only after contact of NK cells with HLA-DR+ bone marrow cells as well as NK-sensitive target cells. However, it is unlikely that the IL 2–induced factor(s) in this case is the same one as NK-CIA because the CFU-C–inhibiting activity was not shown by a coculture of the LGLs with bone marrow cells, but was clearly demonstrated only under the influence of IL 2 administration. Although the precise biochemical characterization of the factor(s) that tends to inhibit CFU-C is under investigation, γ-IFN seems to be greatly involved in the inhibition of granulopoiesis because a high level of γ-IFN was evidently shown not only in culture supernatants but in blood as well. Furthermore, the inhibiting property was mostly abolished by an anti–γ-IFN antibody. Anti–α-IFN had no significant influence on the suppressive effect of the supernatants. IFNs are thought to have interesting effects both on immunoregulation and hematopoiesis, and suppressive ability of γ-IFN in hematopoietic progenitors has been reported by some investigators.[54,55] CFU-C were noted to be more responsive to γ-IFN than other categories of IFNs such as α- (leukocyte) and β- (fibroblast) IFNs.[54] Indeed, spontaneous or mitogen-stimulated γ-IFN production has been demonstrated by lymphocytes from several patients with Tγ-LPD.[14,18,56]

The disease presented in this paper may be termed acute Tγ-LPD because of the rapid clonal expansion of the Tγ lymphocytes. We suggest that such an acute clinical course is related to the unique surface phenotype for a premature NK cell subset and a strong expression of Tac antigen after stimulation of IL 2. Although the lineage of the NK cells in this case still remains to be elucidated, such a leukemialike proliferation of LGLs serves as an experimental model for studying the ontogeny and function of LGLs in both immunoregulation and hematopoiesis. Finally, functional analysis for response to lymphokines as well as immunohistochemical studies of the intracellular expression of γ-IFN and IL 2 may be useful for examining the cell lineage and functional characteristics of abnormal proliferative lymphocytes.

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REFERENCES

1 Herberman RB, Djeu JY, Kay HD, Ortaldo JR, Riccardi C, Bonnard GD, Holden HT, Fagnani R, Santoni A, Puccetti P: Natural killer cells. Characteristics and regulation of activity. Immunol Rev 44:43, 1979

2 Herberman RB, Ortaldo JR: Natural killer cells: Their role in defenses against disease. Science 214:24, 1981

3. Trinchieri G, Perussia B: Human natural killer cells: Biologic and pathologic aspects. Lab Invest 50:489, 1984

4. Holmberg LA, Miller BA, Ault KA: The effect of natural killer cells on the development of syngeneic hematopoietic progenitors. J Immunol 133:2933, 1984

5. Reynolds CW, Foon KA: Tγ-lymphoproliferative disease and related disorders in humans and experimental animals A review of the clinical, cellular, and functional characteristics. Blood 64:1146, 1984

6. Abo T, Miller CA, Gartland GL, Balch CM. Differentiation stages of human natural killer cells in lymphoid tissues from fetal to adult life. J Exp Med 157:273, 1983

7. Abo T, Miller CA, Balch CM: Characterization of human granular lymphocyte subpopulations expressing HNK-1 (Leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. Eur J Immunol 14:616, 1984

8. Ueno Y, Miyawaki T, Seki H, Matsuda A, Taga K, Sato H, Taniguchi N Differential effects of recombinant human interferon-γ and interleukin 2 on natural killer cell activity of peripheral blood in early human development. J Immunol 135:180, 1985

9. Seki H, Ueno Y, Taga K, Matsuda A, Miyawaki T, Taniguchi N: Mode of in vitro augmentation of natural killer cell activity by recombinant human interleukin 2: A comparative study of Leu-11+ and Leu-11− cell populations in cord blood and adult peripheral blood. J Immunol 135:2351, 1985

10. Brouet JC, Flandrin G, Sasportes M, Preud'Homme JL, Seligmann M: Chronic lymphocytic leukaemia of T-cell origin: Immunological and clinical evaluation in eleven patients. Lancet 2:890, 1975

11. McKenna RW, Parkin J, Kersey JH, Gajl-Peczalska KJ, Peterson L, Brunning RD. Chronic lymphoproliferative disorder with unusual clinical, morphologic, ultrastructural and membrane surface marker characteristics. Am J Med 62 588, 1977

12 Bom-van Noorloos AA, Pegels HG, Van Oers RHJ, Silberbusch J, Feltkamp-Vroom TM, Goudsmit TR, Zeiglemaker WP, Von dem Borne AEGKr, Melief CJM: Proliferation of Tγ cells with killer-cell activity in two patients with neutropenia and recurrent infections. N Engl J Med 302:933, 1980

13. Aisenberg AC, Wilkes BM, Harris NL, Ault KA, Carey RW Chronic T-cell lymphocytosis with neutropenia: Report of a case studied with monoclonal antibody. Blood 58:818, 1981

14 Hooks JJ, Haynes BF, Detrick-Hooks B, Diehl LF, Gerrard TL, Fauci AS. Gamma (immune) interferon production by leukocytes from a patient with a T_G cell proliferative disease. Blood 59:198, 1982

15. Starkebaum G, Martin PJ, Singer JW, Lum LG, Price TH, Kadin ME, Paskind WH, Fialkow PJ Chronic lymphocytosis with neutropenia. Evidence for a novel, abnormal T-cell population associated with antibody-mediated neutrophil destruction. Clin Immunol Immunopathol 27:110, 1983

16. Schlimok G, Thiel E, Rieber EP, Huhn D, Feucht H, Lohmeyer J, Reithmuller G: Chronic leukemia with a hybrid surface phenotype (T lymphocytic/myelomonocytic): Leukemic cell displaying natural killer activity and antibody-dependent cellular cytotoxicity. Blood 59:1157, 1982

17. Chan WC, Check I, Schick C, Brynes RK, Kateley J, Winton EF: A morphologic and immunologic study of the large granular lymphocyte in neutropenia with T lymphocytosis. Blood 63:1133, 1984

18. Itoh K, Tsuchikawa K, Awataguchi T, Shiiba K, Kumagai K: A case of chronic lymphocytic leukemia with properties characteristics of natural killer cells. Blood 61:940, 1983

19. Palutke M, Eisenberg L, Kaplan J, Hussain M, Kithier K, Tabaczka P, Mirchandani I, Tenenbaum D: Natural killer and suppressor T-cell chronic lymphocytic leukemia. Blood 62:627, 1983

20 Semenzato G, Pizzolo G, Ranucci A, Agostini C, Chilosi M, Quinti I, De Sanctis G, Vercelli B, Pandolfi F: Abnormal expansions of polyclonal large to small size granular lymphocytes: Reactive or neoplastic process? Blood 63:1271, 1984

21. McKenna RW, Arthur DC, Gajl-Peczalska KJ, Flynn P, Brunning RD. Granulated T cell lymphocytosis with neutropenia. Malignant or benign chronic lymphoproliferative disorder? Blood 66:259, 1985

22. Loughran TP, Kadin ME, Starkebaum G, Abkowitz JL, Clark EA, Disteche C, Lum LG, Slichter SJ: Leukemia of large granular lymphocytes: Association with clonal chromosomal abnormalities and autoimmune neutropenia, thrombocytopenia, and hemolytic anemia. Ann Intern Med 102:169, 1985

23 Newland AC, Catovsky D, Linch D, Cawley JC, Beverley P, San Miguel JF, Gordon-Smith EC, Blecher TE, Shahriari S, Varadi S: Chronic T cell lymphocytosis: A review of 21 cases. Br J Haematol 58:433, 1984

24. Amagasaki T, Shibata J, Yao E, Nishino K, Yamada Y, Sadamori N, Tomonaga M, Kinoshita K, Ichimaru M: A lymphoproliferative disorder of Tγ cells with the phenotype of cytotoxic/suppressor T-cell Am J Hematol 19:85, 1985

25. Costello C, Catovsky D, O'Brien M, Morilla R, Varadi S: Chronic T-cell leukemias Morphology, cytochemistry and ultrastructure. Leuk Res 4:463, 1980

26. Hofman FM, Smith D, Hocking W: T cell chronic lymphocytic leukaemia with suppressor phenotype. Clin Exp Immunol 49:401, 1982

27 Pandolfi F, De Rossi G, Semenzato G, Quinti I, Ranucci A, De Sanctis G, Lopez M, Gasparotto G, Aiuti F: Immunologic evaluation of T chronic lymphocyte leukemia cells. Correlations among phenotype, functional activities, and morphology. Blood 59:688, 1982

28. Pandolfi F, Quinti I, De Rossi G, Frielingsdorf A, Tonietti G, Pasqualetti D, Aiuti F. A population of sheep rosetting cells lacking T- and monocytic-specific antigens, as detected by monoclonal antibodies. Clin Immunol Immunopathol 22:331, 1982

29. Ferrarini M, Romagnani S, Montesoro E, Zicca A, Del Prete GF, Nocera A, Maggi E, Leprini A, Grossi CE: A lymphoproliferative disorder of the large granular lymphocytes with natural killer activity. J Clin Immunol 3:30, 1983

30. Pandolfi F, Pezzutto A, De Rossi G, Pasqualetti D, Semenzato G, Quinti I, Ranucci A, Raimondi R, Basso G, Strong MD, Fontana L, Aiuti F: Characterization of two patients with lymphomas of large granular lymphocytes. Cancer 53:445, 1984

31. Pandolfi F, Semenzato G, De Rossi G, Quinti I, Guglielmi C, Pezzutto A, Lopez M, Tonietti G, Fontana L, Abo T, Aiuti F. HNK-1 monoclonal antibody (Leu-7) in the identification of abnormal expansions of large granular lymphocytes. Clin Exp Immunol 52:641, 1983

32. Catovsky D, Linch DC, Beverly PCL: T cell disorders in haematological diseases Clin Haematol 11:661, 1982

33. Nagasawa T, Abe T, Nakagawa T: Pure red cell aplasia and hypogammaglobulinemia associated with Tγ-cell chronic lymphocytic leukemia. Blood 57:1025, 1981

34 Linch DC, Cawley JC, Worman CP, Galvin MC, Roberts BE, Callard RE, Beverley PCL: Abnormalities of T-cell subsets in patients with neutropenia and an excess of lymphocytes in the bone marrow. Br J Haematol 48:137, 1981

35. Phyliky RL, Li CY, Yam LT: T-cell chronic lymphocytic leukemia with morphologic and immunologic characteristics of cytotoxic/suppressor phenotype Mayo Clin Proc 58:709, 1983

36. Smit JW, Blom NR, van Luyn MJA, Miedema F, Melief CJM, Halie MR T cells in patients with chronic Tγ lymphocytosis: Morphology, cytochemistry, ultrastructure and immunological characteristics. Blut 51:83, 1985

37. Chang TW, McKinney S, Liu V, Kung PC, Vilcek J, Le J:

Use of monoclonal antibodies as sensitive and specific probes for biologically active human γ-interferon. Proc Natl Acad Sci USA 81 5219, 1984

38 Miyawaki T, Seki H, Kubo M, Taniguchi N: Suppressor activity of T lymphocytes from infants assessed by coculture with unfractionated adult lymphocytes in the pokeweed mitogen system. J Immunol 123:1092, 1979

39. Taniguchi N, Miyawaki T, Moriya N, Nagaoki T, Kato E, Okuda N. Mitogenic responsiveness and monocyte-lymphocyte interaction of early and late rosette-forming cell populations of human peripheral blood lymphocytes J Immunol 118:193, 1977

40 Moriya N, Nagaoki T, Okuda N, Taniguchi N: Suppression of adult B cell differentiation in pokeweed mitogen–stimulated cultures by Fc(IgG) receptor–negative T cells from cord blood J Immunol 123 1795, 1979

41. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. J Immunol 126:1393, 1981

42. Suzuki R, Handa K, Itoh K, Kumagai K· Natural killer (NK) cells as a responder to interleukin 2 (IL 2). I. Proliferative response and establishment of cloned cells. J Immunol 130:981, 1983

43 Langford MP, Weigent DA, Georgiades JA, Johnson HM, Stanton GJ: Antibody to staphylococcal enterotoxin A–induced human immune interferon (IFNγ). J Immunol 126.1620, 1981

44 Miyawaki T, Seki H, Taga K, Taniguchi N· Interferon-γ can augment expression ability of HLA-DR antigens on pokeweed mitogen–stimulated human T lymphocytes. Cell Immunol 89:300, 1984

45. Moir DJ, Ghosh AK, Abdulaziz Z, Knight PM, Mason DY· Immunoenzymatic staining of haematological samples with monoclonal antibodies. Br J Haematol 55:395, 1983

46. Koizumi S, Yamagami M, Miura M, Horita S, Sano M, Ikuta N, Taniguchi N Expression of Ia-like antigens defined by monoclonal OKIa1 antibody on hemopoietic progenitor cells in cord blood· A comparison with human bone marrow. Blood 60:1046, 1982

47. Koizumi S, Fine RL, Curt GA, Griffin JD, Chabner BA: Enrichment of myeloid progenitor cells from normal human bone marrow using an immune-rosette technique. Exp Hematol 13.560, 1985

48. Pike BL, Robinson WA. Human bone marrow colony growth in agar-gel. J Cell Physiol 76 77, 1970

49. Rambaldi A, Pelicci PG, Allavena P, Knowles DM II, Rossini S, Bassan R, Barbui T, Dalla-Favera R, Mantovani A: T cell receptor β chain gene rearrangements in lymphoproliferative disorders of large granular lymphocytes/natural killer cells. J Exp Med 162:2156, 1985

50. Yodoi J, Teshigawara K, Nikaido T, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato N, Tsudo M, Uchiyama T, Maeda M: TCGF (IL 2)-receptor inducing factor(s). I. Regulation of IL 2 receptor on a natural killer–like cell line (YT cells). J Immunol 134.1623, 1985

51 Spitzer G, Verma DS, Abramowitz S, Tomasovic B: Cells with Fcγ receptors from normal donors suppress granulocyte macrophage colony formation. Blood 60:758, 1982

52 Hansson M, Beran M, Andersson B, Kiessling R: Inhibition of in vitro granulopoiesis by autologous allogeneic human NK cells. J Immunol 129.126, 1982

53 Degliantoni G, Perussia B, Mangoni L, Trinchieri G: Inhibition of bone marrow colony formation by human natural killer cells and by natural killer cell-derived colony-inhibiting activity. J Exp Med 161:1152, 1985

54 Broxmeyer HE, Lu L, Platzer E, Feit C, Juliano L, Rubin BY: Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. J Immunol 131:1300, 1983

55. Raefsky EL, Platanias LC, Zoumbos NC, Young NS: Studies of interferon as a regulator of hematopoietic cell proliferation. J Immunol 135:2507, 1985

56. Pandolfi F, Capobianchi M, Matricardi P, Facchini J, Bonomo G, De Rossi G, Semenzato G, Fiorilli M, Dianzani F, Aiuti F: Impaired gamma interferon production by cells from patients with lymphoproliferative disorders of mature T and NK cells Scand J Immunol 21 315, 1985

Production of Granulocyte-Macrophage Colony-Stimulating Factor by Abelson Virus–Induced Tumorigenic Mast Cell Lines

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We have recently described a system that supports the development of continuously growing and tumorigenic cell lines after infection of individual multilineage hematopoietic colonies with Abelson murine leukemia virus (A-MuLV). We now provide definitive evidence that these transformed lines express features characteristic of mast cells. Although these lines have been maintained in some cases for more than a year in the absence of exogenous growth factors other than those present in fetal calf serum, colony formation could consistently after 2 months, and variably after 5 months, be shown to be increased several fold when pokeweed mitogen–stimulated spleen cell conditioned medium (CM) was added to the cultures. CM from the A-MuLV–transformed lines was then tested for its ability to stimulate hematopoietic colony formation by cells from both fetal and adult tissues. Four of four randomly selected cell lines produced factors that were active on erythropoietic, granulopoietic, and in some cases pluripotent progenitors. Removal of viral particles from the CM from one of the lines (27d1) by either heat inactivation or high-speed centrifugation did not alter the colony-stimulating activity detected. When CM from 27d1 cells was tested for its ability to stimulate the proliferation of interleukin 3 (IL3) granulocyte-macrophage colony-stimulating factor (GM-CSF)–dependent FDC-P1 cells, a positive result was obtained. This stimulatory activity was not reduced in the presence of neutralizing anti–IL 3 immunoglobulin (Ig), suggesting that the activity detected was GM-CSF and not IL 3. This was confirmed by the lack of expression of the IL 3 gene in 27d1 cells as determined by Northern analysis of 27d1 cell RNA. Furthermore, S1 analysis of mRNA from 27d1 cells as well as two other lines indicated that the GM-CSF gene in all three was transcriptionally active. Taken together, these data suggest that A-MuLV transformation of normal mast cells or their precursors under certain conditions commonly activates the production of GM-CSF.

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ABELSON MURINE LEUKEMIA virus (A-MuLV) is an acute replication-defective transforming retrovirus containing the oncogenic sequence *v-abl*.[1] It is the result of a recombination event between the replication-competent Moloney leukemia virus (M-MuLV) and a part of the host genomic *c-abl*. A-MuLV was originally shown to cause nonthymic B cell lymphoma in vivo,[2] but is now known to cause a variety of hematologic tumor types including plasmacytomas,[3] thymomas,[4] mastocytomas,[5,6] and myelomonocytic leukemia,[7] depending on the specific protocol and route of inoculation. In vitro studies have shown that A-MuLV can transform cells at various stages of the B cell differentiation pathway[8,9] and can do so without blocking B cell differentiation.[9,10] Other in vitro studies have shown that A-MuLV infection of fetal liver cells allowed differentiating erythroid cells to proliferate and mature in the absence of erythropoietin (Ep).[11] These cells, however, could not be passaged into continuously growing lines, indicating that A-MuLV may also alter the requirement of normal differentiating cells for their specific growth factors without their concomitant conversion to a malignant state.

In the murine hematopoietic system, a number of distinct growth factors are now recognized. These were characterized initially on the basis of differences in the progenitor cell populations on which they appeared to act and hence the kinds of colonies whose formation they supported. Several of these so-named colony-stimulating factors (CSFs) have recently been purified to homogeneity and their target cell specificity and functions studied in detail. Three, granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), and macrophage CSF (M-CSF), show specificity for cells differentiating along the granulocyte-macrophage lineage.[12] However, both GM-CSF and G-CSF can also stimulate the limited proliferation of pluripotent progenitors.[13,14] Interleukin 3 (IL 3), also referred to as multi-CSF or by one of several other names, is sufficient to support the full development of a variety of single- and multilineage colonies containing varying combinations of granulocytes, macrophages, eosinophils, megakaryocytes, erythroid cells, and mast cells.[15–17] Recently, the purification of another hematopoietic growth factor, hemopoietin I, has been reported.[18] This latter factor is not itself a CSF, although, when present together with a known CSF, it may allow the production of recognizable progeny derived from primitive but otherwise undetectable hematopoietic progenitor cell types.

The products of several viral oncogenes have been shown to be very similar to normal growth factors or their receptors.[19–21] Naturally arising or induced abnormalities in the structure or expression of the cellular genes that code for these molecules might, therefore, also be expected to contribute to the malignant transformation of cells normally responsive to a particular growth factor. In a previous study, we found that A-MuLV infection of individual multilineage colonies yielded transformed tumorigenic cell lines in high frequency, but only when these were cocultured for the first 2 to 3 months in the presence of a heavily irradiated 3T3 cell

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feeder layer.[22] This suggested that in this system, transformation may involve the acquisition of autonomy from a growth factor produced by irradiated 3T3 cells. To investigate this possibility we examined several of these A-MuLV–transformed cell lines for their sensitivity to exogenous growth factors and for their ability to produce factor(s) with normal hematopoietic cell–stimulating activity.

Preliminary characterization of the cell lines themselves showed that morphologically they resembled mast cells.[22] In this report we provide further evidence of the mast cell characteristics of these cells and demonstrate that they constitutively produce GM-CSF. No IL 3 production could be detected.

MATERIALS AND METHODS

Cells and conditioned media The origin of the transformed lines studied is described elsewhere[22] Briefly, cells from multilineage hematopoietic colonies were removed from methylcellulose cultures and placed together with A-MuLV on top of irradiated 3T3 cells. Cell lines were established independent of feeder cells 2 to 3 months after infection (B1, B2, B9, C3, C8, T6, and 5a), and subsequently, some were cloned (27d1, 27d2). All lines were carried in RPMI 1640 (Flow Laboratories, McLean, Va) supplemented with 20% heat-inactivated fetal calf serum (FCS) and 50 μmol/L 2-mercaptoethanol (2-ME). Conditioned media (CM) from these cell lines were collected three days after feeding cells with fresh medium. Cells were pelleted, and the clarified supernatant was concentrated eight- to tenfold, then stored at −70 °C until used.

Determination of IgE binding and histamine content. Binding of mouse IgE molecules was determined by the method of Kulczycki et al[23] Purified normal mouse mast cells[24] and a CSF-dependent mouse mast cell line, PT-18, cells[25] were used as controls The monoclonal mouse IgE used in the present experiments was the same preparation as that previously described.[26] Cells at a concentration of 1 to 2 × 10^6/mL were suspended in RPMI 1640 medium supplemented with 10% FCS and 0.01 mol/L EDTA, pH 7.6, and incubated with 10 μg/mL ^{125}I-mouse IgE at 37 °C for 90 minutes. A 100-fold excess of unlabeled IgE was added to the cells in the control tubes prior to the addition of ^{125}I-IgE. After incubation, 0.2 mL of the cell suspension was layered over an equal volume of FCS and spun for one minute in a Beckman 152 microfuge (Beckman Instruments, Palo Alto, Calif). The radioactivity of the pellet was counted and the number of ^{125}I-IgE molecules bound per cell calculated assuming a molecular weight of mouse IgE of 184,000.[26]

Histamine was extracted with 1% to 2% perchloric acid and analyzed by the automated technique of Siraganian.[27]

Methylcellulose colony assay. Cells for assays of hematopoietic colony-stimulating activity were obtained either from BALB/c mice from Cumberland View Farm (Tenn) or from BALB/cAnN mice bred and maintained at the NIH (Bethesda, Md). Three different sources of cells were used to assay for CSFs: (1) Fetal peripheral blood was obtained from 9- to 11-day-old embryos, single-cell suspensions prepared, and cells plated at a final concentration of 3 to 4 × 10^4 cells/mL. (2) Bone marrow cells were obtained from normal adult mice and plated at a final concentration of 3 × 10^4 cells/mL. (3) Single-cell suspensions were prepared from the spleens of mice that had received 150 mg/kg 5-fluorouracil (5-FU) intravenously four days before. Cells were plated at a final concentration of 6 to 8 × 10^5 cells/mL.

The culture medium used for colony assays contained 0.8% methylcellulose, 30% FCS (Flow), 1% deionized bovine serum albumin (BSA, Sigma Chemical Co, St Louis), 2 mmol/L L-glutamine and 100 μmol/L 2-ME, and 2 U/mL of Ep. Pokeweed mitogen–stimulated spleen cell conditioned medium (SCM)[28] was included where indicated at a final concentration of 1%. In some cases concentrated CM from A-MuLV–transformed lines were included at a final concentration of 10% (vol/vol) instead of SCM. Colonies were scored in situ under an inverted microscope after six days for embryonic peripheral blood cell cultures and after ten to 12 days for adult bone marrow and spleen cell cultures.

Cell proliferation assays DA-1 cells[29] were obtained from J. Ihle (Frederick Cancer Research Facility, Frederick, Md). FDC-P1 cells[30] were obtained from J. Pierce (National Cancer Institute, NIH, Bethesda, Md). To evaluate the proliferation of these cells, 2 × 10^4 cells in 50 μL of RPMI plus 10% FCS were placed in each well of a 96-well microtiter plate. Appropriate dilutions of IL 3 or CM were then added and the plate incubated for 22 hours at 37 °C. One μCi of ^{3}H-thymidine (Amersham Corp, Arlington Heights, Ill) in 20 μL of medium was then added to each well and the plates incubated for an additional six hours. Cellular DNA was harvested onto glass fiber filter paper and the amount of ^{3}H-thymidine incorporation determined To measure inhibition of factor-induced cell proliferation, wells were set up in the same way with the additional inclusion of an anti–IL 3 immunoglobulin preparation (anti–IL 3 Ig, kindly provided by J. Ihle) in the final culture medium.

Molecular analyses High–molecular weight DNA preparations from A-MuLV-transformed lines, WEHI-3 cells, and BALB/c mouse thymus were prepared[31] and approximately 10 μg of DNA digested with *Eco*RI, then electrophoresed, transferred to nitrocellulose, and hybridized to ^{32}P-labeled IL 3 cDNA probes Two different IL 3 probes were used. One was a 0.8-kb *Pst*I fragment representing the 3′ end of IL 3 cDNA (from J Ihle). The other was a full-length IL 3 cDNA from K. Arai (DNAX, Palo Alto, Calif). After hybridization, the filter was washed for half an hour with 2 × sodium chloride–sodium citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) and then at 65 °C for one hour with 0.1 × SSC/0.1% SDS. The total RNA from A-MuLV–transformed cells, WEHI-3 cells, as well as pokeweed mitogen–stimulated spleen cells was extracted[31] and about 15 μg per lane electrophoresed, transferred, and hybridized to the same probes described. S1 analysis was carried out as described by Ley et al.[32]

Assay for focus-forming units. The titers of A-MuLV in the virus stock and in the concentrated CM were measured using the procedure described by Scher and Siegler[33] Briefly, early-passage 3T3 cells were seeded in 60-mm plastic Falcon dishes (10^5/dish) (Falcon Labware, Oxnard, Calif) in medium with 10 μg/mL of Polybrene The next day the medium was replaced with 0.5 mL viral stock or CM and incubated at 37 °C for one hour with occasional rocking of the dishes. Medium was changed again after five days and transformed foci scored on the tenth day using an inverted microscope.

RESULTS

Mast cell characteristics of A-MuLV–transformed cell lines. Table 1 shows the results of toluidine blue staining, histamine content determinations, and IgE binding studies of cells from two clones (27d1 and 27d2) of a typical A-MuLV–transformed cell line. Comparison of these to fresh and cultured mast cells from another source showed all to be similar. An additional four lines tested were shown to contain histamine, although the amount varied slightly (data not shown). The amount of ^{125}I-IgE that bound to the 27d1 and 27d2 cells was similar to what bound to normal mouse peritoneal mast cells and the IL 3–dependent PT-18 mast cell line (Table 1). Under the same conditions we have found

Table 1. Characterization of A-MuLV–Transformed Mast Cells

Cells	Toluidine Blue	Histamine per 10^6 Cells	IgE Bound per Cell	
			Before Wash	After Wash*
27d1	+	332 ng	2.91×10^5	2.33×10^5
27d2	+	328 ng	2.19×10^5	1.97×10^5
PT-18[26]	+	275 ng	3.33×10^5	3.30×10^5
Mouse mast cells†	+	10.3 µg	3.59×10^5	3.31×10^5

*After incubation with ^{125}I-IgE for 90 minutes, the cells were washed three times with RPMI 1640 medium and cell-bound radioactivity counted.

†Mouse peritoneal mast cells were obtained from BALB/c mice and purified as described elsewhere.[24]

that other cell lines show no binding. These include a cloned line of inducer T lymphocytes (C1, Ly1 + 2 − /9), YAC lymphoma cells,[34] and 33 of 34 lymphocyte cell lines with natural killer (NK)-like or CTL activity.[35] The majority of IgE molecules bound to the A-MuLV–transformed 27d1 and 27d2 cells remained after washing, indicating that the receptors on these cells had a high affinity for the immunoglobulin.

Effect of SCM on the cloning efficiency of A-MuLV–transformed cell lines. The plating efficiency of these lines was studied at various times after their separation from the 3T3 feeders. As shown in Table 2, cells removed from feeders 2 months after infection gave rise to very few colonies when plated in methylcellulose cultures containing FCS but no feeders or any other exogenous source of specific growth factors (eg SCM). At this time, the plating efficiency was increased three- to fourfold if either partially purified IL 3 (obtained as described by Iscove[36]) or crude SCM was added to the assay medium. At later passages, higher plating efficiencies were sometimes encountered, and a difference in colony number was no longer consistently observed when cultures with or without SCM were compared. For example, experiment 2 in Table 2 shows results for some of the same lines used in experiment 1 assayed 3 months later. It can be seen that in this experiment the plating efficiency was approximately 100-fold higher than in experiment 1 and the previously observed effect of added SCM was not seen.

Demonstration of hematopoietic growth factor activity. To assay for colony-stimulating activity, we first tested the ability of concentrated CM from these transformed mast cell lines to stimulate progenitors present in the peripheral blood of 9- to 11-day old mouse embryos. As indicated in Table 3, CM from two A-MuLV–transformed cell lines stimulated the formation of large erythroid colonies, erythroid-mixed colonies, and a number of large nonerythroid colonies consisting mostly of granulocytes and/or macrophages. CM from two other lines (B9 and B2) supported the formation of large erythroid and nonerythroid colonies, but no erythroid-mixed colonies were detected in these assays.

Waneck and Rosenberg[11] have reported that Abelson virus itself can stimulate early hematopoietic progenitor cells from fetal liver to produce macroscopic colonies containing large numbers of differentiating erythroid as well as myeloid elements in the absence of exogenous growth factors. Since the transformed cell lines being examined here were productively infected by A-MuLV[22] (Table 3), we reexamined the colony-stimulating activity present after treatment of CMs with heat or high-speed centrifugation to remove viral particles. Results for 27d1 cell CM are shown in Table 3. It can be seen that a 1,000-fold reduction in viral particles did not significantly alter the colony-stimulating activity of this CM. Neither the number nor the size or erythroid cell content of the colonies obtained appeared to have been affected. Addition of an A-MuLV stock with a titer roughly equivalent to that of the 27d1 cell CM showed some colony-stimulating activity, but the erythroid colonies stimulated were small and were eliminated when the viral stocks were inactivated by heating. The nonerythroid colonies observed after heat inactivation of the A-MuLV stock were probably due to GM-CSF produced by the virus-infected 3T3 cells and present in the virus stocks as reported by Koury and Pragnell.[37]

Similar results were obtained when normal bone marrow cells or spleen cells from 5-FU–treated mice were tested in methylcellulose cultures containing Ep but no other known growth factor (Table 4). Colony formation was again

Table 2. Effect of SCM on the Cloning Efficiency of A-MuLV–Transformed Lines

Lines	Experiment 1		Experiment 2	
	+ SCM	− SCM	+ SCM	− SCM
C8*	(113 ± 18.3) 0.45%	(42.3 ± 5.9) 0.17%	—	—
B9*	(69 ± 9.5) 0.28%	(27.6 ± 4.4) 0.11%	(213.7 ± 9.8) 42.7%	(229 ± 9.7) 45.8%
B1*	(40.7 ± 7.0) 0.16%	(15 ± 2.5) 0.06%	(179 ± 13.5) 35.8%	(169.3 ± 7.0) 33.9%
C3	—	—	(179.3 ± 5.6) 35.9%	(160 ± 5.5) 32%
T6	(6.3 ± 1.2) 0.03%	(1.7 ± 1.2) 0.01%	—	—

*A partially purified SCM was used (see text).

A-MuLV–transformed cells were plated in 1% methylcellulose in α–minimum essential medium (α-MEM) with 36% FCS, 1.2% BSA, 100 µmol/L, 2-ME, and 2mmol/L L-glutamine. Experiment 1 was plated 2 months after virus infection at a final concentration of 5×10^4 cells/mL, whereas experiment 2 was plated 5 months after viral infection at 10^3 cells/mL. The numbers in parentheses are mean values ± SEM where the mean is the average colony number obtained per 0.5 mL of culture

Table 3. Colony-Stimulating Activity of Four A-MuLV–Transformed Cell Line CMs When Added to Cultures of Peripheral Blood Cells from 9- or 11-Day Old Embryos

Conditioned Medium	FFU/ML	Large Erythroid Burst	Erythroid-Mix Colonies	Nonerythroid Colonies
SCM	ND	25 ± 10.2	26.2 ± 5.8	77 ± 3.7
B9*	ND	44 ± 8	<3	191 ± 45
B2*	ND	50 ± 8	6 ± 1	166 ± 25
T6*	ND	33 ± 4	<3	257 ± 25
27d1	3×10^4/mL	25.9 ± 5.3	16.4 ± 4.9	146.6 ± 28.8
Δ 27d1	<20/mL†	17.3 ± 8.2	14.6 ± 5.1	106.8 ± 9.6
27d1 (spun)	40/mL	21.0 ± 6.4	13.3 ± 2.2	86.9 ± 19.8
A-MuLV	8.6×10^4/mL	7.8 ± 3.0	0.36 ± 1.1	20.9 ± 4.7
Δ A-MuLV	<20/mL†	<1.4†	<1.4†	14.6 ± 5.2
R20	ND	<2.6†	<2.6†	<2.6†

Abbreviations: ND, not determined; Δ, heat inactivation by treating at 56 °C for one hour; spun, supernatant collected after centrifugation at 40,000 rpm for one hour; R20, tenfold concentration of RPMI with 20% FCS.

Embryonic peripheral blood cells from 11-day-old embryos (except where noted *, in which case, blood cells from 9-day-old embryos were used) were cultured as described in Materials and Methods. CM from various sources were tested at a final concentration of 10%, except T6 CM, which was tested at a final concentration of 20%.

†Maximum value if only one colony was observed in one experiment.

Colonies were scored six days after initiation of the cultures using the following criteria: large erythroid colonies, containing >300 hemoglobinized erythroblasts; erythroid-mix colonies, containing >200 hemoglobinized erythroblasts and other cell types including granulocytes and macrophages; nonerythroid colonies, containing >50 cells with no hemoglobinized erythroblasts and consisting mainly of granulocytes and/or macrophages. Values shown are means ± SEM from data pooled from two to five experiments and are expressed as the number of colonies scored per 10^5 cells.

observed when either treated or untreated 27d1 CM was added. The number of macroscopic multi-lineage colonies stimulated was, however, relatively lower in normal bone marrow cell cultures than in 5-FU spleen cell cultures.

Absence of IL 3 but production of GM-CSF. We next sought to determine whether 27d1 CM contained IL 3. The effect of 27d1 CM on DA-1 cells (initially thought to be exclusively IL 3 dependent) was therefore examined. As positive controls WEHI-3 cell CM, SCM, and pure IL 3 (from WEHI-3 cell CM)[38] were also tested. These were all capable of stimulating DA-1 cells to proliferate (data not shown). As further indicated in Table 5, addition of anti–IL 3 Ig to wells containing pure IL 3 resulted in a significant inhibition of IL 3–induced proliferation. CM from 27d1 cells was also found to stimulate DA-1 cells, and this activity was not affected by heat inactivation of the virus present in the CM. However, in contrast to the results obtained with pure IL 3, the addition of anti–IL 3 Ig had no effect on the stimulatory activity of 27d1 cell CM.

We next examined the IL 3 gene itself and looked for evidence of its expression in a number of our A-MuLV–transformed cell lines. Figure 1 shows a Southern blot of *Eco*R1-digested high–molecular weight DNA from normal BALB/c mice, WEHI-3 cells, and the 27d1 cell line. The blot was hybridized with a 0.8 kb-*Pst*I fragment representing the 3′ end of the cDNA of the IL 3 coding region (from J. Ihle). As found previously by others, the IL 3 gene was seen to be altered in WEHI-3 cells.[39] However, in 27d1 cells as in normal mouse cells, no alteration was detected. The same result was observed when the blot was hybridized with a full-length IL 3 cDNA probe. Two other A-MuLV–transformed lines gave similar results (data not shown). Figure 2 shows the results of Northern analysis using the 0.8-kb *Pst*I fragment of IL 3 cDNA. A strong band about 1.0

Table 4. Effect of 27d1 Conditioned Media on Colony Formation in Cultures of Adult Bone Marrow and 5-FU–Treated Spleen Cells

Conditioned Medium	BFU-E	CFU-GEMM		CFU-C	
	BM	BM	5-FU Spleen	BM	5-FU Spleen
SCM	15.7 ± 5.7	10.4 ± 2.2	6.2 ± 2.3	187.5 ± 14.9	13.8 ± 5.1
27d1 CM	7.9 ± 2.6	2.7 ± 1.0	8.3 ± 1.0	235.9 ± 13.4	25.9 ± 6.4
Δ 27d1 CM	3.7 ± 1.4	3.8 ± 1.7	7.3 ± 1.6	214.2 ± 12.9	28.1 ± 6.4
27d1 CM (spun)	4.5 ± 1.0	0.32 ± 0.3	8.3 ± 1.1	214.3 ± 12.9	26.6 ± 5.2
A-MuLV	5.0 ± 2.7	<1.0*	<2.3*	47.9 ± 12.4	1.7 ± 0.8
Δ A-MuLV	<1.2*	<1.2*	<2.3*	67.8 ± 19.5	0.2 ± 0.2
R20	3.4 ± 1.7	<1.6*	<2.3*	7.4 ± 4.0	<2.3*

Abbreviations: BFU-E, producing a hemoglobinized erythroid colony consisting of more than three clusters of erythroblasts; CFU-GEMM, producing a colony containing erythroblasts, macrophages, granulocytes, and/or megakaryocytes; CFU-C, producing all nonerythroid colonies containing >50 cells, primarily macrophages, granulocytes, or macrophages and granulocytes.

*Maximum value if only one colony was observed in each experiment.

Colonies were scored under an inverted microscope 10 to 14 days after initiation of cultures from bone marrow (BM) and 5-FU-treated spleen cells. Values given are means ± SEM from data pooled from two to four experiments and are expressed as the number of colonies scored per 10^5 and 10^6 cells for bone marrow and 5-FU-treated spleen, respectively.

Table 5. Effect of CM on the Proliferation of IL 3–Dependent DA-1 Cells

CM	Cell Proliferation		
	CM Alone	CM + Anti-IL 3*	CM + Rb Ig†
IL-3‡	40,411 ± 2,186	29,799 ± 1,272	39,420 ± 45
10% 27d1 CM	47,857 ± 656	46,395 ± 299	47,117 ± 2,386
10% Δ 27d1 CM	45,688 ± 861	40,715 ± 1,437	38,459 ± 2,828
10% A-MuLV	11,646 ± 854	—	—
10% R20	8,253 ± 514	—	—
R10§	9,536 ± 1,530	—	—

Data are presented as means ± SEM. Numbers are ^{3}H-thymidine incorporation into cellular DNA measured in counts per minute.

*The amount of anti-IL 3 Ig added was sufficient to neutralize one third of the input IL 3.

†An amount of normal rabbit Ig equivalent to that present in the anti-IL 3 containing wells was used.

‡Pure IL 3 at a final dilution of 1/200,000.

§ = RPMI 1640 medium supplemented with 10% FCS.

kb in size was observed in lanes containing mRNA from both WEHI-3 cells and pokeweed mitogen–stimulated mouse spleen cells. No band could be detected in the 27d1 cell mRNA. Similar results were again obtained with two other lines (data not shown). Thus it appears that the IL 3 gene is not involved in the generation of any of the activities detected in the CM of our A-MuLV–transformed cell lines.

Since GM-CSF has been reported to stimulate the initial division of pluripotent hematopoietic progenitors and since some IL 3–dependent cell lines can respond to GM-CSF,[40] we looked also for evidence of GM-CSF production by our A-MuLV–transformed cell lines. Figure 3 indicates the results of S1 analysis of RNA from three of the lines. The probe used was the XhoI-*Taq*I fragment in the pCD vector containing the full length cDNA of GM-CSF.[41] This fragment contains 258 nucleotides of the 5′ end of GM-CSF cDNA (Fig 3). RNA from pokeweed mitogen–stimulated spleen cells and from all three transformed mast cell lines (27d1, 27d2, and 5A) examined contained the 258-bp protected fragment, indicating the presence of GM-CSF mRNA. The same size fragment could not be detected in WEHI-3 cells, which do not produce GM-CSF, even when as high as 100 μg of RNA was used for hybridization (Fig 3).

We then tested the 27d1 CM for its ability to stimulate FDC-P1 cells, which have been previously shown to be responsive to GM-CSF as well as IL 3.[40] The results presented in Fig 4 indicate that FDC-P1 cell proliferation was stimulated by 27d1 CM and this stimulation was not reduced by the addition of neutralizing anti–IL 3 Ig. In contrast, the level of stimulation by pure IL 3 was markedly reduced when anti–IL 3 Ig was added to the medium. Additional titration studies indicated that the amount of 27d1 CM used in Fig 4 was equivalent to the amount of IL 3 added in terms of measured thymidine incorporation (data not shown). A slight stimulation of thymidine incorporation seen with increasing amounts of anti–IL 3 Ig or normal rabbit Ig (Fig 4) was also seen without added IL 3 or CM (data not shown).

DISCUSSION

In an earlier paper,[22] we described a method for transforming murine hematopoietic cells with A-MuLV in vitro

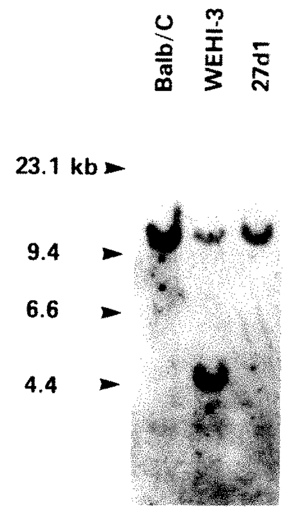

Fig 1. Southern analysis of *Eco*RI-digested high-molecular weight DNA extracted from left to right: BALB/c mouse thymus, WEHI-3 cells, and 27d1 cells. The blot was hybridized to a 0.8-kb *Pst*I fragment of an IL 3 cDNA probe (3′ end). An extra band of about 4.4 kb was observed only with WEHI-3 cells.

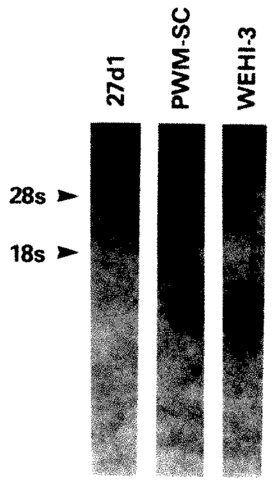

Fig 2. Northern analysis of total RNA extracted from left to right: 27d1 cells, PWM-stimulated spleen cells (PWM-SC), and WEHI-3 cells. The blot was hybridized to the same probe as in Fig 1. A prominent 1.0-kb band was observed with PWM-SC and WEHI-3 RNA, but no band could be detected in 27d1 RNA.

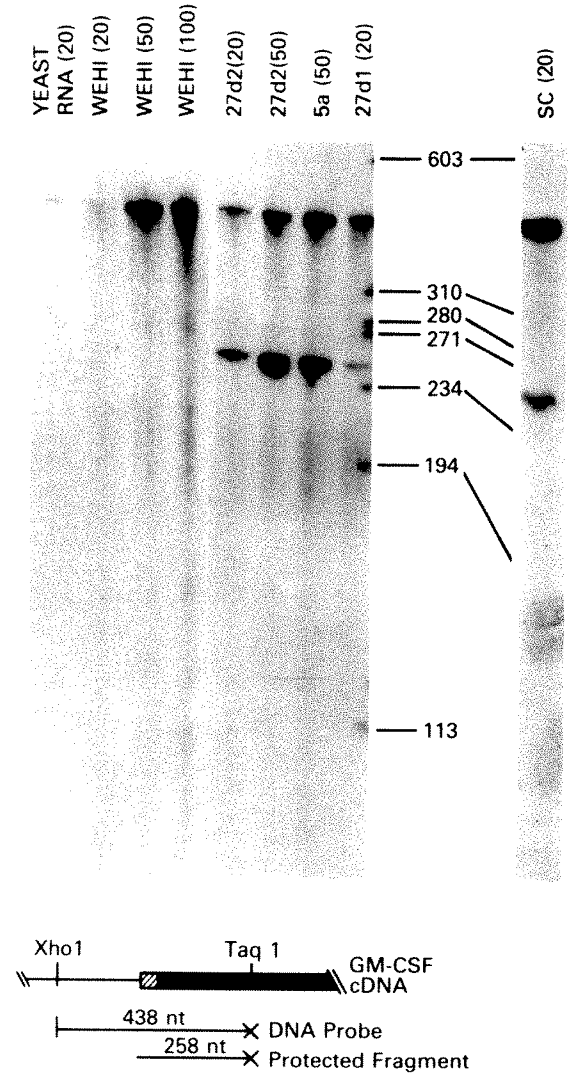

Fig 3. S1 analysis of total RNA extracted from various cell lines from left to right: yeast tRNA, WEHI-3 cells, 27d2 cells, 5a cells, 27d1 cells, molecular weight markers, and pokeweed mitogen-stimulated spleen cells (SC). Numbers in parentheses indicate the amounts of total RNA (in micrograms) used for hybridization. (Bottom) The XhoI-*Taq* I fragment (438 base pair [bp]) containing the 5′ end of GM-CSF cDNA was used as the probe. The protected fragment was 258 bp in size.

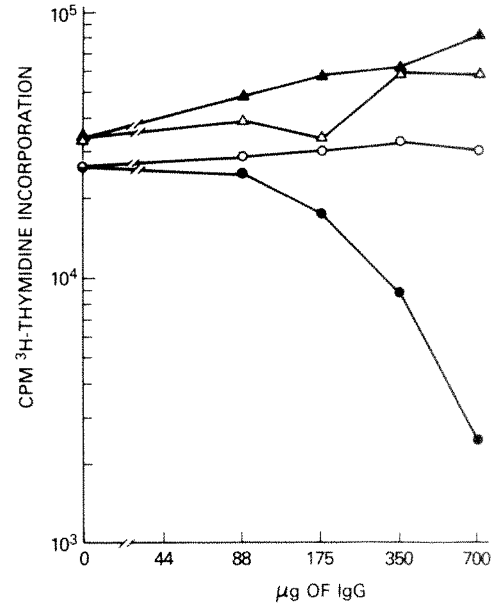

Fig 4. Effect of varying amounts of anti-IL 3 Ig (closed symbols) or normal rabbit Ig (open symbols) on the proliferation of FDC-P1 cells stimulated by 27d1 CM (1.25%, triangles) or 0.25 units of pure IL 3 (circles). Background without any factor added (ie, the medium control) was 2,346 ± 215 cpm.

that allows the rapid emergence of tumorigenic cell lines that morphologically resemble mast cells. In the present study we provide definitive evidence that these cells express mast cell characteristics. Although we showed previously that these lines arose from cells in multilineage colonies of established single-cell origin, this protocol did not allow identification of the type of cell initially infected and transformed. Since 10% to 20% of the cells present in the type of multilineage colonies selected were morphologically identifiable as mast cells, it is possible that mast cells or their precursors were the primary targets. More recent preliminary findings suggest that early hematopoietic progenitors may be targets and that the subsequent phenotype to emerge may be influenced by the conditions used for the selection of transformants (Wong PMC, Chung SW, Raefsky E, Eaves CJ, and Nienhuis AW, unpublished observations). In any case our data confirm the recent finding that mast cells are related to cells of the myeloid lineages.[42] The availability of rapidly growing mast cell lines and a reproducible method for their generation should be useful for future investigations of the development and differentiation of mast cells.

Recently several groups have also reported the isolation of tumorigenic factor-independent mast cell lines following A-MuLV infection of hematopoietic cells in vitro.[43-45] However, in none of these has evidence of production of a hematopoietic growth factor been found. In our experiments, an irradiated 3T3 cell feeder rather than an exogenous supply of IL 3 was used initially to select A-MuLV-transformed lines capable of continuous growth. Because, during early passages, these lines showed an increased plating efficiency in the presence of factors produced by spleen cells, we undertook experiments to investigate more extensively the factor-producing properties of these A-MuLV-transformed cells. CM from four randomly selected cell lines were found to contain activities that stimulated erythroid as well as granulocyte-macrophage and in some cases also multilineage colony formation in methylcellulose assays of murine progenitors from a variety of embryonic and adult sources. However, Northern blots and immunologic analysis indicated that these activities were not attributable to IL 3. From S1 analyses, we found that in three of three lines studied GM-CSF mRNA was produced at a relatively high level (100 copies/cell). Additional evidence of GM-CSF release was obtained when we tested the ability of the CM from one cell line to stimulate the proliferation of FDC-P1 and DA-1 cells. FDC-P1 cells are known to be responsive to GM-CSF as well as IL 3, and we have recently found that

DA-1 cells are also responsive to pure recombinant murine GM-CSF (Wong PMC, Chung SW, Broder T, and Nienhuis AW, unpublished data; and Krystal G, Farber N, and Eaves CJ, unpublished data). Since the FDC-P1 stimulatory activity present in the transformed cell CM was not neutralized by anti–IL 3 Ig, it seems likely that it was GM-CSF. These findings differ most strikingly from those reported by Cook et al[44] who found little or no GM-CSF to be produced by the tumorigenic cells obtained following A-MuLV infection of FDC-P1 cells. Whether differences between the target cells involved or differences in the conditions used to allow A-MuLV transformants to emerge account for these differences in growth factor expression must await further study.

In summary, we have clearly documented the production of at least one hematopoietic growth factor (ie, GM-CSF) by tumorigenic mast cell lines derived from A-MuLV–transformed hematopoietic cells. Because these cells could at an early stage in their development be shown to respond to factors present in pokeweed mitogen–stimulated spleen cell conditioned media, it is possible that their ability to produce factor(s) themselves, which we detected subsequently, may have played a role in the early evolution of a malignant phenotype. Although such a model awaits further study, it is interesting to note that the conversion of FDC-P1 cells to GM-CSF production by retroviral transfer of the GM-CSF cDNA sequence was recently shown to confer autonomous growth and tumorigenic potential on these cells.[46]

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REFERENCES

1. Baltimore D, Rosenberg N, Witte ON: Transformation of immature lymphoid cells by Abelson murine leukemia virus. Immunol Rev 48:3, 1979

2. Abelson HT, Rabstein LS: Lymphosarcoma: Virus-induced thymic-independent disease in mice. Cancer Res 30:2213, 1970

3. Potter M, Sklar MD, Rowe WP: Rapid viral induction of plasmacytomas in pristane-primed BALB/c mice. Science 182:592, 1973

4. Cook WD: Rapid thymomas induced by Abelson murine leukemia virus. Proc Natl Acad Sci USA 79:2917, 1982

5. Mendoza GR, Metzger H: Disparity of IgE binding between normal and tumor mouse mast cells. J Immunol 117:1573, 1976

6. Risser R, Potter M, Rowe WP: Abelson virus–induced lymphomagenesis in mice. J Exp Med 148:714, 1978

7. Raschke WC, Baird S, Ralph P, Nakoing I: Functional macrophage cell lines transformed by Abelson leukemia virus. Cell 15:261, 1978

8. Rosenberg N, Baltimore D: A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. J Exp Med 143:1453, 1976

9. Whitlock CA, Ziegler SF, Treiman LJ, Stafford JI, Witte ON: Differentiation of cloned populations of immature B cells after transformation with Abelson murine leukemia virus. Cell 32:903, 1983

10. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, Coffman R, Rosenberg N, Tonegawa S, Baltimore D: Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J 3:1209, 1984

11. Waneck GL, Rosenberg N: Abelson leukemia virus induces lymphoid and erythroid colonies in infected fetal cell cultures. Cell 26:79, 1981

12. Metcalf D: The granulocyte-macrophage colony-stimulating factors. Science 229:16, 1985

13. Metcalf D, Johnson GR, Burgess AW: Direct stimulation by purified GM-CSF of the proliferation of multipotent and erythroid precursor cells. Blood 55:138, 1980

14. Metcalf D, Nicola NA: Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. J Cell Physiol 116:198, 1983

15. Ihle JN, Keller J, Oroszlan S, Henderson L, Copeland T, Fitch F, Prystowsky MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B: Biological properties of homogenous interleukin 3: 1. Demonstration of WEHI-3 growth factor activity, P-cell stimulating activity, colony stimulating activity and histamine producing cell stimulating factor activity. J Immunol 131:282, 1983

16. Greenberger JS, Humphries RK, Messner H, Reid DM, Sakakeeny MA: Molecularly cloned and expressed murine T-cell gene product is biologically similar to interleukin-3. Exp Hematol 13:249, 1985

17. Rennick DM, Lee FD, Yokata T, Arai K, Cantor H, Nabel G: A cloned MCGF cDNA encodes a multi-lineage hematopoietic growth factor: Multiple activities of interleukin-3. J Immunol 134:910, 1985

18. Jubinsky PT, Stanley ER: Purification of hemopoietin 1: A multilineage hemopoietic growth factor. Proc Natl Acad Sci USA 82:2764, 1985

19. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasterson A, Westermark B, Heldin CH, Huang JS, Deuel TF: Platelet-derived growth factor is structurally related to the putative transforming protein P28^{sis} of simian sarcoma virus. Nature 304:35, 1983

20. Robins KC, Antoniades HN, Devare SG, Hunkapiller MW, Aaronson SA: Structural and immunological similarities between simian sarcoma virus gene product(s) and human platelet-derived growth factor. Nature 305:605, 1983

21. Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, Antoniades HN: Simian sarcoma virus *onc* gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221:275, 1983

22. Wong PMC, Humphries RK, Chen TR, Eaves CJ: Evidence for a multi-step pathogenesis in the generation of tumorigenic cell lines from hemopoietic colonies exposed to Abelson virus in vitro. (submitted)

23. Kulczycki A Jr, Metzger H: The interaction of IgE with rat basophilic leukemia cells. II. Quantitative aspects of the binding reaction. J Exp Med 140:1676, 1974

24. Sterk AR, Ishizaka T: Binding properties of IgE receptors on normal mouse mast cells. J Immunol 128:838, 1982

25. Pluznik DH, Tare NS, Zatz MM, Goldstein AL: Mast-basophil cell line dependent on colony stimulating factor. Exp Hematol 10:211, 1982 (suppl 12)

26. Liu FT, Bohn JW, Gerry EL, Yamamoto H, Molinaro CA, Sherman LA, Klinman NR, Katz DH: Monoclonal dinitrophenyl–specific murine IgE antibody: Preparation, isolation and characterization. J Immunol 124:2728, 1980

27. Siraganian RP: An automated continuous flow system for the

extraction and fluorometric analysis of histamine Anal Biochem 57:383, 1974

28. Eaves CJ, Krystal G, Eaves AC: Erythropoietic cells, in Baum SJ (ed): Current Methodology in Experimental Hematology. Basel, Karger, 1984, p 81

29. Ihle JN, Rein A, Mural R: Immunologic and virologic mechanisms in retrovirus-induced murine leukemogenesis, in G Klein (ed). Advances in Viral Oncology, vol 4 New York, Raven, 1984, p 95

30 Dexter TM, Garland JM, Scott D, Scolnick E, Metcalf D: Growth of factor-dependent hemopoietic precursor cell lines. J Exp Med 152:1036, 1980

31 Maniatis T, Fritsh EF, Sambrook J. Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Laboratory, 1982

32. Ley TJ, Anagnou NP, Pepe G, Nienhuis AW: RNA processing errors in patients with β-thalassemia. Proc Natl Acad Sci USA 79:4775, 1982

33 Scher CD, Siegler R. Direct transformation of 3T3 cells by Abelson murine leukemia virus. Nature 253:729, 1975

34. Galli SJ, Dvorak AM, Ishizaka T, Navel G, Simonian HD, Cantor H, Dvorak HF: A cloned cell with NK function resembles basophils by ultrastructure and expressed IgE receptors. Nature 298:288, 1982

35 Galli SJ, Brooks CG, Dvorak AM, Ishizaka T: Lack of detectable IgE receptor expression on 33 of 34 cell lines with NK-like or CTL activity. Cell Immunol (in press)

36. Iscove NN: Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned medium from lectin-stimulated mouse spleen cells, in Golde DW, Cline MJ, Metcalf D, Fox CF (eds): Hematopoietic Cell Differentiation. Orlando, Fla, Academic, 1978, p 37

37. Koury MJ, Pragnell IB: Retroviruses induce granulocyte-macrophage colony stimulating activity in fibroblasts. Nature 299:638, 1982

38 Ihle JN, Keller J, Henderson L, Klein F, Palaszinski E: Procedures for the purification of interleukin-3 to homogeneity. J Immunol 129:2431, 1982

39. Ymer S, Tucker QJ, Sanderson CJ, Hapel AJ, Campbell HD, Young IG: Constitutive synthesis of interleukin-3 by leukemia cell line WEHI-3B is due to retroviral insertion near the gene. Nature 317:255, 1985

40 Hapel AJ, Warren HS, Hume DA. Different colony-stimulating factors are detected by the "Interleukin-3"–dependent cell lines FDC-P1 and 32D cl-23. Blood 64:786, 1984

41. Miyatake S, Otsuka T, Yokota T, Lee F, Arai K: Structure of the chromosomal gene for granulocyte-macrophage colony stimulating factor: Comparison of the mouse and human genes. EMBO J 4:2561, 1985

42. Pharr PN, Suda T, Bergmann KL, Avila LA, Ogawa M: Analysis of pure and mixed murine mast cell colonies. J Cell Physiol 120.1, 1984

43. Pierce JH, DiFiore PP, Aaronson SA, Potter M, Pumphrey J, Scott A, Ihle JN: Neoplastic transformation of mast cells by Abelson-MuLV. Abrogation of IL-3 dependence by a nonautocrine mechanism. Cell 41:685, 1985

44. Cook WD, Metcalf D, Nicola NA, Burgess AW, Walker F. Malignant transformation of a growth factor–dependent myeloid cell line by Abelson virus without evidence of an autocrine mechanism. Cell 41:677, 1985

45. Oliff A, Agranovsky O, McKinney MD, Murty VVVS, Bauchwitz R: Friend murine leukemia virus–immortalized myeloid cells are converted into tumorigenic cell lines by Abelson leukemia virus. Proc Natl Acad Sci USA 82:3306, 1985

46. Lang RA, Metcalf D, Gough NM, Dunn AR, Gonda TJ. Expression of a hemopoietic growth factor cDNA in a factor-dependent cell line results in autonomous growth and tumorigenicity. Cell 43:531, 1985

Adult-Onset Cyclic Neutropenia Is Associated With Increased Large Granular Lymphocytes

By Thomas P. Loughran, Jr, Edward A. Clark, Thomas H. Price, and William P. Hammond

Human cyclic neutropenia occurs in adults as well as children. Clinical illness is similar in the childhood and adult diseases, but distinctly different modes of onset suggest heterogeneity in its pathophysiology. We studied seven patients with cyclic neutropenia, three with disease acquired in adulthood, and four with the childhood-onset disorder. All three patients with adult-onset cyclic neutropenia had increased numbers of circulating large granular lymphocytes (LGL), whereas the four children with cyclic neutropenia had normal LGL counts. LGL from patients with adult-onset cyclic neutropenia expressed cell surface antigens HNK-1 (three of three patients) and IgG Fc receptors (two of three patients), although natural killer activity was low. Two of these patients were treated with alternate-day steroids, resulting in decreased LGL counts and abrogation of neutrophil cycling. We suggest that adult-onset cyclic neutropenia may be distinguished from the childhood-onset form of the disease by increased numbers of LGL. Furthermore, increased LGL may identify a subset of patients with cyclic neutropenia who respond to steroid therapy.

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HUMAN CYCLIC NEUTROPENIA is a rare disorder characterized by regular predictable oscillations of the blood neutrophil count. Fever, malaise, aphthous stomatitis, and mucous membrane infections occur at the time of the neutrophil nadir. Manifestations of this disease usually begin in childhood, although in about 25% of patients the first symptoms occur after age 20.[1,2] Therapy such as androgens and splenectomy has been modestly successful in a few patients but has not reliably resulted in a correction of the cycling.[3–8] Lithium, although abrogating cycling in the grey collie model of cyclic neutropenia,[9,10] has not been uniformly efficacious in human disease.[11–14] Corticosteroid therapy, however, has been shown to correct neutrophil cycling in a few patients with the adult-onset form of the disorder.[15–17]

The etiology of cyclic neutropenia is not known, although the defect appears to reside at the stem cell level.[18–21] Studies in patients with the adult-onset form of this disease may provide insight into the pathogenesis of this disorder. Recently a syndrome of chronic neutropenia associated with excess large granular lymphocytes (LGL) has been identified.[22] We have shown that in some of these patients this disease results from a clonal expansion of LGL.[23] In this report, we describe an association between excess numbers of circulating LGL and the adult-onset form of cyclic neutropenia.

MATERIALS AND METHODS

Patients All patients with the exception of patient 3 were referred to us for evaluation of neutropenia and were studied at the Clinical Research Center at the University Hospital on protocols approved by the Human Subjects Review Committee of the University of Washington. Patient 3 was recruited for study through the National Institutes of Health (NIH) Clinical Center: slides of bone marrow obtained prior to steroid therapy were obtained from the NIH, whereas blood for surface marker studies was obtained through her private physician.

All three patients with adult-onset cyclic neutropenia had recurrent symptoms of fever, malaise, aphthous stomatitis, and occasional local infections. Clinical onset of disease in patient 1 began in 1970 at the age of 59 when a complete blood cell count (CBC) showed neutropenia and absolute lymphocytosis. CBC with differential count had been normal in 1968 when she underwent distal pancreatectomy and splenectomy for chronic pancreatitis The diagnosis of cyclic neutropenia was established in 1975. A trial of lithium carbonate in 1979 was unsuccessful, as described previously.[11] She continues to experience periodic fevers, malaise, and aphthous stomatitis; she has declined treatment with alternate-day prednisone. Patient 2 was well until 1962 when at the age of 45 symptoms began to occur at approximately monthly intervals A diagnosis of cyclic neutropenia was established in 1975, and a trial of alternate-day prednisone (50 mg) resulted in correction of neutrophil cycling. She currently remains well, receiving 20 mg prednisone every other day Cyclic neutropenia was diagnosed in patient 3 at the age of 65. She has been the subject of previous studies of cyclic neutropenia performed at the NIH.[1,15,24] A trial of alternate-day prednisone resulted in abrogation of neutrophil cycling and the clinical manifestations of her disease.[15] She still receives prednisone, 10 mg daily, and has suffered multiple complications of steroid therapy including bleeding gastric ulcer, osteoporosis resulting in fracture of the right hip, and diabetes mellitus

Patients 4 to 7 with childhood-onset cyclic neutropenia also had classic features of the disease: regularly recurring episodes of fever, malaise, aphthous stomatitis, and cervical lymphadenopathy. Patients 4 and 6 were previously reported by us[11]; patient 4 died of necrotizing clostridial enterocolitis at age 11. Patient 5 presented with a lung abscess; she had severe periodontitis, as did patient 6 All four patients with childhood-onset cyclic neutropenia had cycle lengths between 19 and 21 days, all had monocytosis during neutropenia, and three had cyclic fluctuations of either platelet or reticulocyte counts.

Patients 8 to 10 with chronic neutropenia had histories of recurrent infections similar to those of patients with cyclic neutropenia. Patients 8 and 10 were referred by their physicians because of the severity of their infections. They had otherwise normal blood counts and fit the diagnostic category of chronic idiopathic neutropenia.[25] Patient 9 was also anemic and thrombocytopenic; he had had documented neutropenia and severe recurrent skin infections for at least 10 years These patients served as one control group, whereas ten healthy volunteers served as the normal control group

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Blood counts. The diagnosis of cyclic neutropenia was established by performing blood counts a minimum of three times per week for at least 6 weeks as previously described.[1,2,11] Patients with chronic neutropenia had serial blood counts similarly performed for a minimum of 4 weeks to document the absence of cycling. WBC counts were done on EDTA-anticoagulated specimens using a Coulter counter and total neutrophil counts (bands plus neutrophils) calculated from the WBC and a 100-cell differential count. LGL were identified by their characteristic morphology on Wright-Giemsa stains, ie, larger size and smaller nuclear cytoplasmic ratio than typical lymphocytes as well as prominent azurophilic granules.[23] Total LGL counts were calculated by multiplying the total lymphocyte count by the percentage of LGL (of at least 100 cells counted) obtained in the differential lymphocyte count. Differential counts were performed by one observer (T.P.L.) on coded specimens to prevent biased reporting. Five hundred-cell bone marrow differential counts were performed by a single observer (T.H.P.).

Lymphocyte studies Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and studied for expression of cell surface antigens using an Epics 5 flow cytometer with direct one-color and two-color analysis using a panel of fluorescein-conjugated or R-phycoerythrin–conjugated monoclonal antibodies (MoAb) as previously described.[26] The antigens recognized by these MoAb are described using the current World Health Organization international nomenclature.[27] The following MoAb, verified by International Workshop analyses, were kindly provided by Dr Jeff Ledbetter (Genetic Systems, Seattle): G19-4 anti-CD3 (T3) and G10-1 anti-CD8 (T8). Dr Toru Abo (University of Alabama, Birmingham) provided HNK-1.[28] The Fc-1 MoAb recognizes IgG Fc receptors (CD16) found on natural killer (NK) cells and does not recognize IgG Fc receptors expressed on monocytes [26] NK activity of peripheral blood mononuclear cells was determined using a ^{51}Cr-labeled K562 erythroleukemia cell line as a target in a four-hour assay as previously described for our laboratory.[29] Effector:target ratios were 6.25:1, 12.5:1, 25:1, and 50:1; lytic units for 30% killing of 10^4 target cells were derived from a graph of $\log_{10}$ effector/target ratio *v* the percent specific lysis plotted on semilog paper. Cytogenetic studies were done on peripheral blood mononuclear cells from patients 1 and 3 cultured either with or without phytohemagglutinin as previously described.[23]

Neutrophil antibody studies Tests for serum leukoagglutinating antibodies were performed according to the method of McCullough et al [30] Neutrophil-associated immunoglobulin and serum or plasma neutrophil–binding immunoglobulin were determined by indirect immunofluorescence using a modification of the method of Verheught et al [31] Patient or normal target neutrophils were purified by Hypaque-Ficoll sedimentation and fixed with 1% paraformaldehyde. Neutrophil-associated immunoglobulin (direct test) was determined by flow microfluorometry after incubation of the cells with fluorescein isothiocyanate (FITC) goat antihuman IgG (or IgM) (Tago, Burlingame, Calif). Results were quantitated using peak fluorescence channel numbers and expressed as a multiple of average values for normal neutrophils. For determination of plasma/serum neutrophil-binding immunoglobulin (indirect test), normal neutrophils were incubated with test sera, washed, and surface immunoglobulin determined as before. For the indirect test, results are expressed as a multiple of values seen when target cells are incubated with autologous serum.

RESULTS

Cyclic neutropenia was well documented by frequent serial blood counts in seven patients. Of the patients with adult-onset cyclic neutropenia, patient 1 was studied on several occasions over a 10-year period; cycle length has remained constant at 20 days.[11] Patient 3 has also been studied on several occasions over at least 14 years; cycle length has remained constant at 21 to 22 days.[1,15,24] Blood counts obtained on patient 2 over a 10-month period before and after receiving prednisone therapy are shown in Fig 1. The cycle length was somewhat longer, with an average of 27 days. Alternate-day prednisone therapy eventually resulted in correction of cycling and stabilization of neutrophil counts at levels greater than 2,000/μL. Similar results of prednisone therapy have been reported previously for patient 3.[15]

All three patients with adult-onset cyclic neutropenia had a marked increase in circulating LGL at the time of diagnosis (Table 1), values similar to those observed in patients with LGL leukemia.[23] To determine whether an elevated LGL count was a nonspecific finding secondary to neutropenia and recurrent infections, we determined LGL counts in other groups of neutropenic patients. As shown in Table 1, three patients with chronic neutropenia and four children with cyclic neutropenia had LGL counts within the normal range. Clinical remission of adult-onset cyclic neutropenia was associated with a reduction in numbers of LGL in the two patients treated with alternate-day steroids (Table 1). Results of bone marrow differential counts in patients with adult-onset cyclic neutropenia are shown in Table 2. All three patients had increased numbers of mature-appearing marrow lymphocytes. In addition, characteristic findings of cyclic neutropenia were seen in serial marrow examinations from patient 1. During neutropenia, myeloid precursor cells predominated, whereas during the neutrophil recovery phase an expansion of the myeloid postmitotic pool was observed. Of interest, microscopic examination of the spleen of patient 1, obtained prior to clinical onset of cyclic neutropenia, revealed lymphoid infiltration of splenic red pulp cords, a finding similar to that reported in patients with LGL leukemia.[23]

Figure 2 shows serial LGL, lymphocyte, and neutrophil counts obtained from patient 1 during a 26-day period

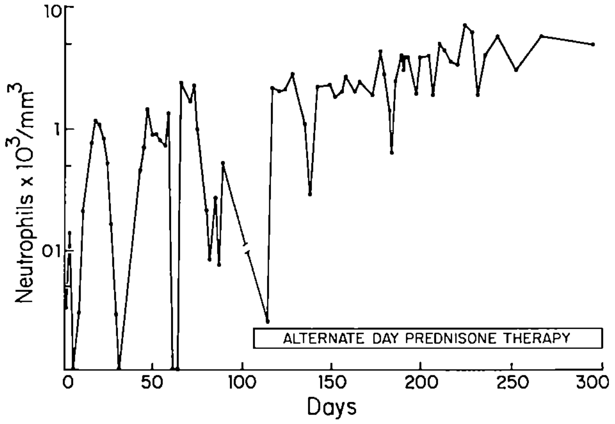

Fig 1. Serial neutrophil counts obtained on patient 2 over a 300-day period are plotted using a log scale. Period length of neutrophil cycling was 27 days, determined by dividing the total number of days (109) by the number of cycles observed (four). As indicated by slashed lines, no data were obtained between days 89 and 114. Alternate-day prednisone therapy resulted in a rise in the neutrophil nadir and eventully in normalization of the neutrophil count.

Table 1. Clinical and Hematologic Features of Neutropenic Patients

Patient	Age	Sex	WBC	Neutrophils	Lymphocytes	Percentage of LGL	Total LGL	Clinical Features
Adult-onset cyclic neutropenia								
1	74	F	9,900	0–1,984*	8,415	73	6,143	Severe fever, aphthous stomatitis, malaise
2								
Untreated	68	F	3,900	0–2,000	3,100	70	2,170	Recurrent skin infections, fever, aphthae
Steroid therapy			7,600	5,092	1,672	62	1,037	
3								
Untreated	80	F	7,800	0–3,600	4,900	83	4,067	Fever, skin infections, aphthous stomatitis
Steroid therapy			9,700	6,402	1,843	63	1,161	
Childhood-onset cyclic neutropenia								
4	11	M	2,500	25–2,682	1,380	16	221	Died of clostridial sepsis and enterocolitis
5	19	F	5,500	0–1,353	3,465	13	450	Right lung abscess, severe periodontal disease
6	10	M	3,900	0–1,628	1,860	16	298	Otitis media, aphthous stomatitis
7	6	F	5,500	0–1,944	4,015	9	361	Otitis media, fever
Chronic neutropenia								
8	20	F	2,800	244–902	2,016	6	121	Occasional skin infections
9	22	M	2,500	243–945	1,750	4	70	Severe recurrent skin infections, glomerulitis
10	21	F	2,500	73–1,402	1,472	7	103	Severe aphthous stomatitis
Normals†								
n = 10	37 ± 14	5M/5F	5,920 ± 1,999	3,654 ± 1,563	2,221 ± 832	11 ± 6	223 ± 99	
	(22–58)		(3,100–9,500)	(1,333–5,035)	(1,300–4,275)	(5–24)	(104–482)	
n = 42	—	27M/15F	—	—	—	14 ± 4	245 ± 108	
	(14–80)					(5–29)	(120–620)	

*Ranges indicate fluctuations from nadirs to peak values

†Normal values were obtained in our laboratory on ten healthy volunteers The top line gives the mean ± SD, the bottom line gives the range in parentheses LGL values are compared with those obtained on 42 healthy controls in another laboratory [32]

WBC, neutrophils, lymphocytes, and total LGL are cells/μL Hematocrit and platelet counts were normal in all patients except patient 9, whose hematocrit value was 31% and platelet count was 104,000 cells/μL

encompassing two nadirs in neutrophil counts. Fluctuations in the total lymphocyte count occurred in parallel to that observed in LGL count. In contrast to the regular periodicity observed in the neutrophil counts, however, there was no definite cycling in either LGL or total lymphocytes in patient 1, although sufficient data were not available to perform a periodogram analysis on this patient. Such an analysis previously done in patient 3, however, did reveal cycling of the patient's total lymphocytes[24] now shown, retrospectively, to have been mostly LGL.

Surface marker studies of peripheral blood mononuclear cells from all three patients revealed some phenotypic characteristics of NK cells. All patients had increased numbers of HNK-1+ cells; in addition, two patients had LGL that expressed IgG Fc receptors (Table 3). Two-color analysis showed that HNK-1+ cells from patient 1 coexpressed CD3 and CD8 and lacked expression of IgG Fc receptors (CD16). Results of two-color analyses of peripheral blood mononuclear cells from patients 2 and 3 obtained while both patients were receiving steroid therapy were more heterogeneous. In both patients the majority of HNK-1+ cells lacked expression of either CD3, CD8, or CD16, although 20% to 43% of HNK-1+ cells did express these other markers (Table 3). In spite of elevated numbers of mononuclear cells bearing NK-associated antigens, the NK activity in all three patients was markedly reduced (<0.001 lytic units for patients 1 to 3, compared with 5.5, 10.4, and 2.2 lytic units obtained for normal concurrent controls for patients 1 to 3, respectively). Cytogenetic studies showed normal karyotypes in phytohemagglutinin-stimulated peripheral blood mononuclear cell cultures from patient 1 and in unstimulated peripheral blood mononuclear cell cultures from patient 3. There were no analyzable metaphases in unstimulated peripheral blood mononuclear cell cultures from patient 1.

Results of neutrophil antibody studies are shown in Table 4. Leukoagglutination studies were negative in all three

Table 2. Bone Marrow Differential Counts

Counts	Patients				Normal*
	1 (No. 1)	1 (No. 2)	2	3	
Erythroid	9.8	15.2	27.4	34.0	15.0–36.2
Myeloblast	0.4	0.2	1.3	1.9	0.1–1.7
Promyelocyte	7.8	0.9	1.7	3.5	1.9–4.7
Myelocyte	1.2	6.5	1.5	8.5	8.5–16.9
Metamyelocyte	0.9	4.3	0.9	4.4	7.1–24.7
Bands + segments	0.9	24.6	6.3	8.5	13.2–26.4
Lymphocytes	61.9	39.9	46.5	29.4	8.6–23.8
Other	17.1	8.4	14.4	9.8	—
Blood PMN	0	1,540	900	NA	

Abbreviations: NA, not available; PMN, polymorphonuclear cells

*A 95% confidence range.[33]

Results expressed as a percentage, 500 cells counted

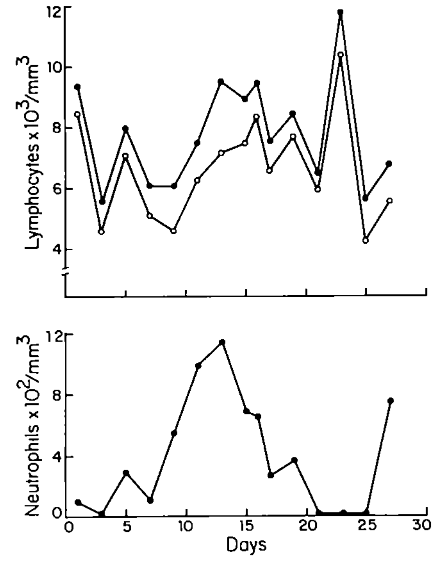

Fig 2. The top graph depicts serial total lymphocyte counts (black circles) and LGL counts (white circles) obtained from patient 1 during one period of neutrophil cycling (bottom graph). Although there were fluctuations in both total lymphocytes and LGL, a regular periodicity similar to that of neutrophils was not observed.

patients. There was no evidence for circulating antineutrophil antibodies in either patient 1 or 3; in contrast, both IgG and IgM antibodies were demonstrated in the serum of patient 2. High levels of neutrophil-associated IgM were documented in patient 2; this patient also had elevated serum levels of IgM (808 mg/dL). In addition, there was a slight increase in neutrophil-associated IgG in patient 3. Of note, patient 3 had previously been shown to have a normal neutrophil survival.[24]

DISCUSSION

This study suggests that adult-onset cyclic neutropenia may be distinguished morphologically from the more common childhood-onset cyclic neutropenia by the presence of increased numbers of circulating LGL. All three patients with the adult-onset disorder met clinical and hematologic criteria for cyclic neutropenia: (1) recurrent symptoms of fever, malaise, aphthous stomatitis, and occasional superficial infections and (2) periodic episodes of profound neutropenia associated with reciprocal monocytosis. In patients 1 and 3 the cycle length was within the 20- to 22-day cycle period seen in 85% of patients with cyclic neutropenia,[1] whereas the cycle length in patient 2 was a bit longer (27 days). Furthermore, the cycle lengths of individual patients were very stable when studied at intervals of several years, a feature of classic cyclic neutropenia.[1,2]

Table 3. Expression of NK Cell–Associated Antigens on Peripheral Blood Mononuclear Cells of Patients With Adult-Onset Cyclic Neutropenia (Percentage Positive)

	Patient			
Antigens	1	2	3	Normal*
HNK-1+ total	46	54	60	13 ± 3.9
HNK-1+,CD3(T3)+	35	11	16	2 ± 0.8
HNK-1+,CD8(T8)+	41	14	17	3 ± 1.6
HNK-1+,CD16(IgG FcR)+	1	23	20	4 ± 2.4
CD16+ total	5	34	30	13 ± 6.4

*Normal values shown represent the mean ± SD obtained from four normal controls.

Table 4. Neutrophil Antibody Results

		Immunofluorescence			
		Direct*		Indirect†	
Patient	Lag	IgG	IgM	IgG	IgM
1	negative	ND	ND	0.5	0.9
2	negative	1.5	5.9	2.9	10.3
3	negative	2.0	0.9	0.8	0.6
Normal‡	—	0–1.9	0–1.4	0–2.4	0–1.8

Abbreviation: Lag, leukoagglutination; ND, not determined

*Results expressed as multiples of values for control cells.

†Results expressed as multiples of values for autologous serum.

‡A 95% confidence limit.

Results of lymphocyte surface antigen phenotyping in these patients with acquired cyclic neutropenia revealed some heterogeneity. Mononuclear cells from the only patient studied in an untreated state (patient 1) were CD3+, CD8+, HNK-1+, CD16−. The other two patients also had increased numbers of HNK-1+ cells; approximately 40% of these cells also expressed IgG Fc receptors, a surface antigen normally associated with active NK cells.[34–37] Of note, NK activity of peripheral blood mononuclear cells from all three patients was very low, a finding previously noted in most patients with large granular lymphocytosis and chronic neutropenia.[22,23]

No prior studies of LGL in adult-onset cyclic neutropenia have been reported. Lymphocyte subpopulations have been studied in three children with cyclic neutropenia,[12,38] but LGL were examined in only one of these patients.[38] In that report, LGL (defined as HNK-1+ cells) fluctuated from 0.7% to 7.5% of the total blood lymphocytes; using this data, we estimate that absolute LGL numbers were always below 300/ μL, consistent with our finding of normal LGL counts in the childhood disorder.

The syndrome of chronic neutropenia associated with excess LGL was first described in 1977 and has recently been reviewed.[22] We showed previously that in at least some of these patients this disorder represents a leukemia of LGL that is characterized by autoimmune manifestations and infiltration of splenic red pulp, hepatic sinusoids, and bone marrow by immature NK cells.[23] Although cycling of neutrophil counts did not occur in patients with LGL leukemia (unpublished observations), the three patients with adult-onset cyclic neutropenia we report here do share features of that disease: (1) excess LGL that express some NK surface antigens yet have little NK activity and (2) lymphocytic infiltration of splenic red pulp cords and possibly bone marrow. In contrast, our patients with adult-onset cyclic neutropenia did not have arthritis or positive tests for antinuclear antibody or rheumatoid factor, common features of LGL leukemia.[23,39] Furthermore, they have had extraordinarily prolonged illnesses ranging from 8 to 14 years, demonstrating a remarkable stability of the disorder.

The etiology of cyclic neutropenia is not known. Previous marrow transplantation studies have suggested that this disorder is a result of a periodic failure of myelopoiesis occurring at the stem cell level.[18–21] However, it may not be possible to generalize these studies performed in the autosomal recessive model disease of grey collie dogs or in human childhood-onset cyclic neutropenia to the adult-onset disease in humans. Kinetic studies, though, have shown normal neutrophil survival, fluctuating neutrophil reserves, and virtually absent myelocyte incorporation of ^{3}H-thymidine during neutrophilia in both childhood-onset and adult-onset disease in humans,[1,24] strongly suggesting a common final pathway for the neutropenia, namely periodic failure of production. In patients with LGL leukemia, on the other hand, there is some evidence that neutropenia is secondary to autoimmune peripheral destruction.[23,40] In contrast to these findings, not all of our patients with adult-onset cyclic neutropenia had antineutrophil antibodies. Furthermore, normal neutrophil half-life was demonstrated previously in patient 3.[24] These data suggest that the mechanism of neutropenia in patients with adult-onset cyclic neutropenia may differ from that observed in patients with LGL leukemia.

Perhaps of most interest was the dramatic clinical response to alternate-day steroid therapy. Both patients 2 and 3 had correction of neutrophil cycling; the other patient declined such treatment. Therapy was associated with stabilization of bone marrow neutrophil precursors and neutrophil marrow reserves in patient 3.[15] Although LGL counts still remained greater than normal, clinical remission induced by prednisone was also associated with a reduction in numbers of LGL. Thus, it seems likely that increased LGL are involved in adult-onset cyclic neutropenia, either as a causative factor or as still another phenomenological component of cyclic fluctuations of stem cells. Whether LGL cycle in all untreated patients would be interesting to know but can not be determined from this study; in any event, the marked elevation of LGL levels in patients with adult-onset disease is in striking contrast to the normal levels in childhood-onset disease. Also of note, a lymphoid infiltration of splenic red pulp cords was documented in patient 1, 2 years before the clinical and hematologic onset of cyclic neutropenia. Furthermore, LGL are known to produce multiple regulatory factors,[41,42] and it has been emphasized that cyclic neutropenia may be secondary to an abnormality in feedback regulation of hematopoiesis.[43]

Two other patients with cyclic neutropenia have been reported to respond to steroid therapy.[16,17] Another patient had a temporary response to plasmapheresis.[44] Documented improvement in neutrophil counts was also observed in at least three patients following splenectomy,[5–7] in one patient undergoing splenectomy and receiving adrenocorticotropic hormone injections,[8] in two patients receiving androgen therapy,[3,4] and in one patient receiving lithium.[45] All of these patients had the adult-onset form of cyclic neutropenia; lymphocyte morphology was not noted. In contrast, no successful therapy for childhood-onset cyclic neutropenia has been reported. The results of our study indicate that adult-onset cyclic neutropenia may be characterized by markedly increased LGL and suggest that the pathogenesis of this form of the disease may be different from the childhood-onset disorder. Furthermore, this form of the disease may be amenable to steroid or other therapy. Future studies on similar patients may provide further insight into the pathogenesis of cyclic neutropenia and the regulatory role of LGL in myelopoiesis.

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REFERENCES

1. Wright DG, Dale DC, Fauci AS, Wolff SM: Human cyclic neutropenia: Clinical review and long-term follow-up of patients. Medicine 60:1, 1981

2 Lange R: Cyclic hematopoiesis. Human cyclic neutropenia. Exp Hematol 11:435, 1983

3. Brodsky I, Reimann HA, Dennis LH: Treatment of cyclic neutropenia with testosterone. Am J Med 38:802, 1965

4. Roozendaal KJ, Dicke KA, Boonzajer Flaes ML: Effect of oxymetholone on human cyclic hematopoiesis. Br J Haematol 47:185, 1981

5. Fullerton HW, Duguid HLD: A case of cyclical agranulocytosis with marked improvement following splenectomy. Blood 4:269, 1949

6 Sandella JF: Cyclic acute agranulocytosis: Report of a case with improvement after splenectomy Ann Intern Med 35:1365, 1951

7. Natelson RP: Cyclic neutropenia with giant follicular lymphoblastoma and lymphosarcoma. Blood 8:923, 1953

8. Monto RW, Shafer HC, Brennan MJ, Rebuck JW: Periodic neutropenia treated by adrenocorticotropic hormone and splenectomy. N Engl J Med 246:893, 1952

9. Hammond WP, Dale DC: Lithium therapy of canine cyclic hematopoiesis. Blood 55:26, 1980

10. Hammond WP, Dale DC: Cyclic hematopoiesis· Effects of lithium on colony-forming cells and colony-stimulating activity in grey collie dogs. Blood 59·179, 1982

11. Hammond WP, Berman B, Wright DG, Dale DC: Lithium is an ineffective therapy for human cyclic hematopoiesis. Blood 61 1024, 1983

12. Borkowsky W, Shenkman L, Rausen A: T-lymphocyte cycling in human cyclic neutropenia: Effects of lithium in vitro and in vivo. Clin Immunol Immunopathol 23 586, 1982

13. Lange RD, Crowder CG, Cruz P, Hawkinson SW, Lozzio CB, Machado E, Painter P, Terry W, Jones JB: Cyclic neutropenia: A tale of two brothers and their family. Am J Pediatr Hematol Oncol 3:127, 1981

14. Ishii E, Hara T, Miyazaki S, Fujiwara T, Goya N: Lithium therapy for cyclic neutropenia in children. Scand J Haematol 31:193, 1983

15. Wright DC, Fauci AS, Dale DC, Wolff SM: Correction of human cyclic neutropenia with prednisolone. N Engl J Med 298:295, 1978

16. Rogers GM, Shuman MC: Acquired cyclic neutropenia and successful treatment with prednisone Am J Hematol 13:83, 1982

17. Young GAR, Iland HJ, Deveridge SF, Forrest PR, Vincent PC: Steroid responsive cyclical neutropenia. Blut 48·153, 1984

18. Dale DC, Graw RG Jr· Transplantation of allogeneic bone marrow in canine cyclic neutropenia. Science 183:83, 1974

19. Weiden PL, Robinett B, Graham TC, Adamson J, Storb R· Canine cyclic neutropenia: A stem cell defect. J Clin Invest 53:950, 1974

20. Jones JB, Yang TJ, Dale JB, Lange RD: Canine cyclic hematopoiesis: Marrow transplantation between littermates. Br J Haematol 30:215, 1975

21. Krance RA, Spruce WE, Forman SJ, Rosen RB, Hecht T, Hammond WP, Blume KG: Human cyclic neutropenia transferred by allogeneic bone marrow grafting. Blood 60:1263, 1982

22. Reynolds CW, Foon KA: T gamma-lymphoproliferative disorders in man and experimental animals· A review of the clinical, cellular, and functional characteristics. Blood 64:1146, 1984

23. Loughran TP Jr, Kadin ME, Starkebaum G, Abkowitz JL, Clark EA, Disteche C, Lum LG, Slichter SJ: Leukemia of large granular lymphocytes: Association with autoimmune neutropenia, thrombocytopenia, and hemolytic anemia. Ann Intern Med 102:169, 1985

24. Guerry D, Dale DC, Omine M, Perry S, Wolff SM: Periodic hematopoiesis in human cyclic neutropenia. J Clin Invest 52:3220, 1973

25. Greenberg PL, Mara B, Steed S, Boxer L: The chronic idiopathic neutropenia syndrome: Correlation of clinical features with in vitro parameters of granulopoiesis Blood 55·915, 1980

26. Ledbetter JA, Tsu TT, Draves K, Clark EA: Differential expression of pan T-cell antigens on human T-cell subsets, in Heise E (ed): Perspectives in Immunogenetics and Histocompatibility. New York, American Society of Histocompatibility Genetics 1985, p 325

27. Bernard A, Bernstein I, Boumsell L, Dawsset J, Erons R, Hansen J, Haynes B, Kersey J, Knapp W, McMichael A, Milstein C, Reinherz R, Ritts RE, Schlossman SF: Differentiation human leukocyte antigens: A proposed nomenclature. Immunol Today 5 158, 1984

28. Abo T, Balch CM: A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1) J Immunol 127·1024, 1981

29. Clark EA, Sturge JC: Phylogeny of NK cell reactivity against human and non-human primate lymphoblastoid cell lines. Evolving and conserved target antigens. J Immunol 126:969, 1981

30. McCullough J, Clay ME, Priest JR, Jensen NJ, Lau S, Noreen HJ, Drivit W, Lalezari P: A comparison of methods for detecting leukocyte antibodies in autoimmune neutropenia. Transfusion 21:483, 1981

31. Verheught FWA, VonDemBorne AEG, DeCary F, Engelfriet CP: The detection of granulocyte alloantibodies with an indirect immunofluorescence test. Br J Haematol 36.533, 1977

32. Gastl G, Niederweiser D, Marth C, Huber H, Egg D, Schuler G, Margreiter R, Brawnsteiner H, Huber C: Human large granular lymphocytes and their relationship to natural killer cell activity in various disease states. Blood 64:288, 1984

33. Lee GR, Boggs DR, Bithell TC, Foerster J, Athens JW, Lukens JN (eds): Clinical Hematology (ed 8). Philadelphia, Lea & Febinger, 1981, p 58

34. Abo T, Miller CA, Balch CM: Characterization of human granular lymphocyte subpopulations expressing HNK-1 (Leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. Eur J Immunol 14:615, 1984

35. Perussia B, Starr S, Abraham S, Fanning V, Trinchieri G: Human natural killer cells analyzed by B73 1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. J Immunol 130.2133, 1983

36. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF: Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 131:1789, 1983

37 Perussia B, Trinchieri G, Jackson A, Warner NL, Fawst J, Rumpold H, Kraft D, Lanier LL: The Fc receptor of IgG on human natural killer cells: Phenotype, functional, and comparative studies with monoclonal antibodies. J Immunol 133:180, 1984

38. Engelhard D, Landreth KS, Kapoor N, Kincade PW, DeBawlt LE, Theodore A, Good RA: Cycling of peripheral blood and marrow lymphocytes in cyclic neutropenia. Proc Natl Acad Sci USA 80:5734, 1983

39. Wallis WJ, Loughran TP Jr, Kadin ME, Clark EA, Starkebaum GA: Polyarthritis and neutropenia associated with circulating large granular lymphocytes. Ann Intern Med 103:357, 1985

40. Starkebaum G, Martin PJ, Singer JW, Lum LG, Price TH, Kadin ME, Raskind WH, Fialkow PJ: Chronic lymphocytosis with neutropenia: Evidence for a novel, abnormal T-cell population associated with antibody-mediated neutrophil destruction. Clin Immunol Immunopathol 27:110, 1983

41. Kasahara T, Djeu JY, Dougherty SF, Oppenheim JO: Capacity of human large granular lymphocytes (LGL) to produce multiple lymphokines: Interleukin 2, interferon, and colony stimulating factor. J Immunol 131:2379, 1983

42 Scala GP, Allavena P, Djeu JY, Kasahara T, Ortaldo JR, Herberman RB, Oppenheim JJ: Human large granular lymphocytes are potent producers of interleukin-1. Nature 309:56, 1984

43. VonSchulthess GK, Mazer NA· Cyclic neutropenia. A clue to the control of granulopoiesis. Blood 59:27, 1982

44 VonSchulthess GK, Fehr J, Dahinden C: Cyclic neutropenia: Amplification of granulocyte oscillations by lithium and long-term suppression of cycling by plasmapheresis. Blood 62:320, 1983

45 Verma DS, Spitzer G, Zander AR, Dicke KA, McCredie KB: Cyclic neutropenia and T lymphocyte suppression of granulopoiesis: Abrogation of the neutropenic cycles by lithium carbonate. Leuk Res 6:567, 1982

Acute Lymphoblastic Leukemia and Non-Hodgkin's Lymphoma of T Lineage: Colony-Forming Cells Retain Growth Factor (Interleukin 2) Dependence

By Ivo Touw, Ruud Delwel, George van Zanen, and Bob Löwenberg

The regulatory role of interleukin 2 (IL 2) in the proliferation of T acute lymphoblastic leukemia (T-ALL) and T non-Hodgkin's lymphoma (T-NHL) cells from six individual patients was analyzed in a colony culture system to which pure recombinant IL 2, and the lectin phytohemagglutinin (PHA) or the phorbol ester 12-0-tetradecanoyl phorbol-13-acetate (TPA), had been added. The proliferative response was correlated with the inducibility of receptors for IL 2 on the surface membrane of T-ALL and T-NHL cells by incubation with TPA or PHA for 18 hours. Leukemic T cell colonies, identified by immunophenotyping or cytogenetic analysis, appeared in vitro following TPA and IL 2 stimulation in all six cases. Accordingly, receptors for IL 2, initially absent from the cell surface, were found on high proportions of the T-ALL and T-NHL cells after in vitro exposure to TPA. In contrast, colony formation stimulated by PHA and the induction of IL 2 receptors by PHA were limited to the one case of T-NHL with the mature thymocyte immunophenotype. The cells from the other patients, expressing common or prothymocyte phenotypes, did not respond to PHA. No colonies were formed in any of these cases when PHA or TPA was withheld from the IL 2-containing cultures. Although colony growth depended absolutely on exogenous IL 2 in three cases (ALL), in the three other cases (one ALL, two NHL) some colonies grew also when no IL 2 had been added to the cultures. Upon further analysis of the cells of one of the latter patients, it was found that the cells produced IL 2 and proliferated in response to this endogenous IL 2. The results from this study indicate that the requirements of endogenous *v* exogenous IL 2 for cell proliferation in T-ALL and T-NHL and IL 2 receptor activation by PHA and TPA vary from patient to patient. In addition, they support the notion that T-ALL and T-NHL cells have not lost dependence on IL 2 and IL 2 receptor activation for in vitro growth.

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ATTEMPTS AT UNDERSTANDING the underlying mechanisms of tumor growth are concerned with the possibility that neoplasms are disturbed in their response to growth factors. Their uncontrolled outgrowth may be caused by an altered sensitivity to growth regulators (eg, as a result of aberrant expression or function of membrane receptors) or to the acquired ability to self-produce factors.[1] These phenomena have been described for a variety of tumors and in a number of cases were found to correlate with the expression of oncogenes.[2-4] Alternatively, proliferation of tumor cells may become completely independent of normal growth regulators.[4]

The availability of interleukin 2 (IL 2) gene probes, pure recombinant IL 2 preparations, and monoclonal antibodies (MoAb) reactive with cell surface membrane receptors for IL 2[5,6] allows for the analysis of the growth requirements of human T cell leukemias. These approaches have been pursued mainly in leukemias of mature T cell type, in particular, those associated with the human T cell lymphotropic virus I (HTLV I).[7] Until now, however, scarce information was available on the control of proliferation by IL 2 in immature type T cell neoplasms, ie, T acute lymphoblastic leukemia (T-ALL) and certain T cell non-Hodgkin's lymphomas (T-NHL)

In the present study, we have examined T-ALL and T-NHL cells from six individual patients for their proliferative response to IL 2. We applied a colony culture technique in which pure recombinant IL 2 (r-IL 2) was added as the single growth factor. The lectin phytohemagglutinin (PHA) or the phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA) was supplemented simultaneously for activation of the cells.

Our findings indicate that distinct patterns of IL 2 and activation requirements can be recognized in individual cases of T-ALL and T-NHL. However, like normal T cells, the leukemia cells remain dependent on TPA or PHA activation and IL 2 for proliferation. The proliferating neoplastic cells do not acquire new surface antigens indicative of maturation to later cell stages, suggesting that their capacity to differentiate in vitro is very limited. We conclude that proliferation of T-ALL and T-NHL cells cannot be simply explained by the loss of control by IL 2 or by the aberrant (ie, independent of activation) expression of membrane receptors for IL 2.

MATERIALS AND METHODS

Patient data and immunologic phenotypes of leukemic T cells Selected clinical and immunologic data of the patients entered into this study are summarized in Table 1 Patients with prothymocytelike (stage I) leukemic cells (TdT+, WT1+, E+/−, T3−, T4−, T6−, T8−) had been diagnosed cytohistopathologically as cases of T-ALL (patients 1 to 3) and patients carrying thymocyte-like (stage II, stage III) malignant T cells (T3−, T6+, T4/T8+ or T3+, T4+, T10+, T6−, T8−) as T-ALL (patient 4) or leukemic T-NHL (patients 5 and 6).[8,9]

Isolation of leukemic T cells. Leukemic cells were isolated from peripheral blood or bone marrow at diagnosis, after informed consent, using Ficoll-Isopaque separation.[10] A further separation was applied to remove residual normal (E+, T3+) T lymphocytes because, in most cases, these could interfere with leukemic growth in the IL 2-supplemented cultures In one case of E rosette-negative T-ALL (ie, patient 1), normal T cells were eliminated by E rosette depletion using 2-aminoethylthiouronium bromide (AET)-treated sheep erythrocytes as described.[1]

In the other case (ie, patients 2 through 5), residual normal T lymphocytes were removed from the leukemic cell fractions on the basis of differential T3 antigen expression on normal and neoplastic

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Table 1. Selected Data of Patients With T-ALL and T-NHL

Patient	Age	Sex	Diagnosis	Blood Leukocyte Count (× 10^{-9}/L)	Leukemic Immunophenotype* (Percentage of Positive Cells)					
					WT1†	T3	T4	T6	T8	E
1	8	F	T-ALL	180	98	0	0	0	0	0
2	4	F	T-ALL	100	45	0	0	0	0	75
3	10	M	T-ALL	200	100	W	7	3	13	93
4	30	M	T-ALL	53	98	0	34	98	96	92
5‡	19	M	T-NHL	40	ND	0	34	37	64	80
6§‖	63	M	T-NHL	166	96	98	94	0	0	95

Abbreviation: ND, not determined

*Assessed by indirect immunofluorescence and E rosetting (200 cells counted).

†Weak expression (see Fig 1).

‡Diffuse, lymphoblastic lymphoma.

§Eighty-eight percent of the cells expressed T10 antigens

‖Diffuse, well-differentiated lymphocytic lymphoma.

Patients 1 to 5 are terminal deoxynucleotidyl transferase (TdT)-positive; patient 6 is TdT-negative. In none of the cases did the leukemic cells express Ia antigens.

T cells by fluorescent staining of the T3 antigen (see the subsequent sections) followed by fluorescence-activated cell sorting (FACS 440, Becton Dickinson, Sunnyvale, Calif) (Fig 1). The cells of patient 6 were studied without further separation because both cytogenetic and immunologic analysis of fresh and cultured cells indicated that contaminating growth of normal T cells did not occur.

Isolation of E rosette-forming lymphocytes from normal peripheral blood samples. E rosette-forming lymphocytes were recovered from the sediment after centrifugation of AET-treated E rosetted cells through Ficoll-Isopaque as described.[11] Sheep erythrocytes were removed from these fractions by hypotonic lysis (incubation for five minutes at 37 °C in 0.75% wt/vol NH_4Cl in 3 mmol/L Tris-HCl, pH 7.2).

Immunofluorescence. Surface membrane antigens on fresh and cultured cells were assayed by indirect immunofluorescence microscopy using murine MoAb and goat antimouse Ig immunoglobulins coupled to fluorescein isothiocyanate (GAM/FITC; Nordic Immunology, Tilburg, The Netherlands).[12] The presence of TdT in the cell nuclei was assessed on cytocentrifuged cells using an indirect immunofluorescence kit (GIBCO, Ghent, Belgium).

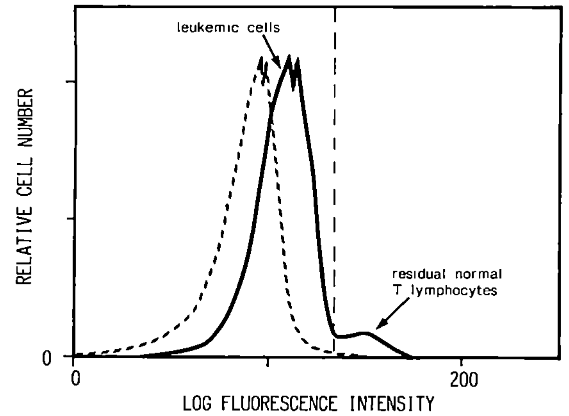

Fig 1. FACS histograms of Ficoll-Isopaque isolated cells of patient 3 (T-ALL) after OKT3 + GAM FITC (——) and GAM/FITC control (---) staining. The ALL cells of this patient showed a weak T3 antigen expression, whereas residual normal T lymphocytes (interfering strongly with leukemic colony growth) express the T3 antigens in a high density on their cell surface. To eliminate the normal T cells from the ALL cell sample of this patient (and of patients 2, 4, and 5) cell sorting was performed on the basis of T3 fluorescence intensity. The vertically drawn interrupted line indicates the level at which the cells were separated; ALL cells were recovered left from this line (the fraction with low fluorescence intensity).

MoAb. MoAb reactive with the T3, T4, T6, T8, and T10 antigens and with the Ia antigen were obtained from Ortho Pharmaceutical Corp (Raritan, NJ) and all used in a 1:40 dilution. MoAb WT1, reactive with a pan-T cell antigen[13] was supplied by Dr W.J.M. Tax (Nijmegen, The Netherlands) and used in a titer of 1:100 MoAb anti-IL 2 receptor (aIL 2r) obtained from Becton Dickinson (Mountain View, Calif) was used in a 1:20 dilution. The reactivity of this antibody is identical to that of anti-Tac.[14] MoAb B13.9, detecting a myelocytic antigen, was a gift from Dr P. Lansdorp (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Colony culture. Colony cultures were performed basically as described for normal T cell colony formation[15] with the following modifications: (1) Feeder leukocytes were not included in the system as a source of growth stimuli. Instead, pure r-IL 2 (Biogen SA, Geneva) was added to the culture upper layer. (2) Either PHA (reagent grade, Wellcome Reagents, Dartford, England) at a concentration of 0.7% vol/vol or the phorbol ester TPA at a concentration of 7 ng/mL (Sigma Chemical Co, St Louis) were used as mitogens in culture.

Colony cells were mass harvested with a Pasteur pipette, washed three times with phosphate-buffered saline (PBS), and prepared for indirect immunofluorescence and E rosette formation.

In one case (patient 6), the colony cells were analyzed cytogenetically (Dr A. Hagemeyer, Department of Cell Biology and Genetics, Erasmus University, Rotterdam).

Induction of IL 2 receptors. Short-term (18 hours) suspension cultures supplemented with PHA or TPA to induce membrane receptors for IL 2 were performed as described.[16] After culture, the cells were washed three times with PBS and prepared for indirect immunofluorescence with the aIL 2r antibody.

Microculture of NHL cells and in vitro blocking of IL 2 receptors. Proliferation of NHL cells in culture as measured by ^{3}H-thymidine (TdR) uptake was examined with and without blocking of IL 2 receptors. Parallel cultures were established to which MoAb aIL 2r (IgGl, noncytotoxic), control MoAb (OKT3, B13.9) or no MoAb had been added at their initiation A quantity of 1×10^5 cells were cultured in triplicate in round-bottom microtiter wells (Greiner, Alphen an den Rijn, The Netherlands) for three days in 100 μL RPMI 1640 medium (GIBCO) with 10% heat-inactivated fetal calf serum supplemented with 5×10^{-5} mol/L beta-mercaptoethanol and L-glutamine (referred to as complete RPMI medium) supplemented with 0.1% PHA.

Sixteen hours before harvesting of the cells (Titertek Cell Harvester 550) 0.1 μCi of TdR (Amersham Corp, Amersham, UK; specific activity, 2 Ci/mmol) was added to each microwell. Radioactivity was measured by liquid scintillation counting (Beckman LS 3800, Beckman Instruments, Inc, Fullerton, Calif). All cultures were performed in triplicate. Data are expressed as mean dpm ± SD of triplicate cultures.

IL 2 assay. Murine IL 2–dependent cytotoxic T lymphocyte cell line (CTLL) cells (a gift from S Knaan-Shanzer, Radiobiological Institute TNO, Rijswijk, The Netherlands) were cultured in triplicate after three washings with Hanks' balanced salt solution (HBSS) in complete RPMI medium at 1×10^5 cells/100 μL in microtiter wells. At 20 hours, cultures were supplemented with 0.1 μCi TdR, and four hours later, the cells were harvested and assayed for TdR incorporation by liquid scintillation counting.

To estimate IL 2 concentration in T-NHL culture supernatant, the proliferation of CTLL cells in the presence of these supernatants was compared with proliferation in response to titrated r–IL 2 concentrations (ranging from 0.01 to 100 U/mL).

Preparation of T-NHL culture supernatant. A quantity of 2×10^6 cells T-NHL cells/mL complete RPMI medium with or without 1% vol/vol PHA in 6-mL tubes (Greiner) was placed in the incubator for three days. Supernatants were then recovered, filter sterilized through a 0.22-μm filter (Millipore, Bedford, Mass), and stored at −20 °C.

RESULTS

Induction of IL 2 receptors. Untreated T-ALL and T-NHL cells, like normal peripheral blood T lymphocytes, did not express membrane receptors for IL 2 as measured in indirect immunofluorescence using MoAb aIL 2r (Table 2). Studies on cells from short-term (18 hours) suspension cultures then disclosed that IL 2 receptors appeared on significant proportions of the cells when TPA or PHA had been added to these cultures (Table 2). In the absence of TPA/PHA, IL 2 receptor induction did not occur. A striking discrepancy with respect to PHA sensitivity was evident between the prothymocyte or immature thymocytelike leukemia cells (ie, patients 1 to 5) and the normal T lymphocytes. These leukemias, in contrast to the normal T cells, did not respond to PHA. In contrast, the cells of patient 6 carrying a mature thymocyte phenotype were sensitive to activation by both TPA and PHA, thus sharing this property with normal peripheral T cells.

Colony stimulation. To assess the proliferative response of the normal T and the T-NHL/T-ALL cells to IL 2, the cells were plated in colony culture under selected stimulatory conditions (Table 2).

Colony formation by the normal T lymphocytes depended on the presence of both IL 2 and mitogen (either PHA or TPA). When the addition of mitogen (ie, PHA or TPA) was omitted from IL 2–containing cultures, no colonies were formed, which is in agreement with the finding that nonactivated T cells lack membrane receptors for IL 2. On the other hand, suboptimal colony formation was noted in the presence of TPA or PHA when no IL 2 had been added to the cultures. The fact that some normal T cell colonies develop in non–IL 2–supplemented cultures is most likely to be explained by endogenous production of IL 2 by the T cells stimulated by the IL 1–like activity of TPA[17] and/or some residual monocytes present among the cells.

The colony-forming abilities of the cells from patients 1 to 5 were clearly different from those of normal blood T lymphocytes. The cells did not form colonies in response to PHA even when IL 2 had been added to culture. In contrast, when combined with TPA, IL 2 stimulated the formation of considerable numbers of colonies in all of these cases. The capacity of the cells to form colonies in response to TPA but not to PHA was entirely in accordance with, respectively, the ability and inability of these compounds to induce IL 2 receptors in the individual cases. The cells of patient 6 showed a divergent response pattern: colony formation and IL 2 receptor induction occurred with either TPA or PHA.

To define the IL 2 dependence of T-ALL and T-NHL colony-forming cells, IL 2 dose titration experiments were performed (Fig 2). These experiments show that the T-ALL cells of patients 1 and 3, activated by TPA, like normal T lymphocytes require the addition of IL 2 to the cultures for optimal colony formation (panels A, B, and D). Patient 1 is representative of the pattern in which the cells are typically absolutely dependent on the exogenous supply of IL 2 to culture. This type of response also applies to that in patients 2

Table 2. Induction of IL 2 Receptors and Colony Formation

	IL 2 Receptor Expression (Positive Cells)			Colony Formation† (Different Stimulatory Conditions)					
Patient*	Percentage of Untreated	TPA‡	PHA‡	TPA + IL 2 25	TPA	PHA + IL 2	PHA	IL 2 25	None
1	0	97	0	76	0	0	0	0	0
2	0	47	0	30	0	0	0	0	0
3	3	61	5	47	36	0	0	0	0
4	0	20	0	14	0	0	0	0	0
5	5	18	2	35	23	8	0	0	0
6	0	22	95	41	0	166	106	0	0
Normal T§	0	55	24	ND	ND	539	88	0	0
Normal T§	0	26	55	410	266	872	72	0	0

*See Table 1 for a description of the patients.

†Colony numbers per 2×10^6 plated cells

‡Determination of IL 2 receptors following 18 hours of TPA/PHA-supplemented suspension culture (see Materials and Methods).

§E-positive lymphocytes purified from normal donor peripheral blood

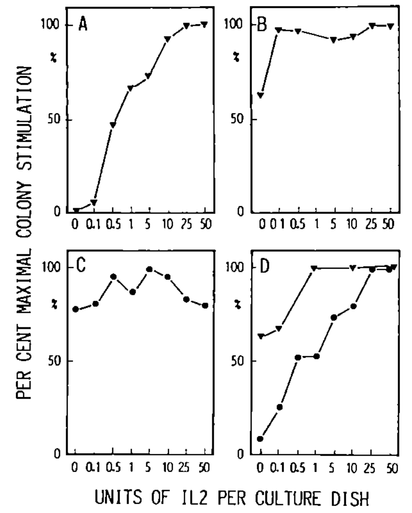

Fig 2. Induction of ALL/NHL (A, B, C) and normal (D) T colony formation with titrated doses of r–IL 2. The data are expressed as percentages of maximal colony stimulation. (A) T-ALL patient 1. (B) T-ALL patient 3. (C) T-NHL patient 6. (D) E+ lymphocytes purified from normal peripheral blood: ▼—▼, TPA costimulation; ●—●, PHA costimulation.

and 4. Plateau IL 2 requirements for leukemic colony formation were variable, ie, 25 units per culture dish for patient 1 and 0.1 units per dish for patient 3. Further increases in IL 2 concentrations (up to 500 units per culture dish) did not significantly alter the numbers of (normal and leukemic) colonies.

The TPA-activated T-ALL cells of patient 3 produced colonies (although in suboptimal numbers) without the addition of IL 2. A similar phenomenon was evident with the T-NHL cells of patient 6 cultured with PHA (panel C). These findings raise the possibility that certain T-ALL and T-NHL cells are capable of IL 2 release and autostimulation (see the following).

Colony typing. Membrane marker analysis was performed after colony culture (Table 3), and the immunophenotypes of colony cells were compared with those of the fresh cells.

Immunologic phenotypes of the leukemic colony cells in general reflected similar stages of maturation as the cells before culture. For example, colony cells from patients 1, 2, and 3 still expressed the surface antigens characteristic of their preculture immaturity (Table 1).

Colony cells of patients 4 and 5 appeared slightly more mature than the cells before culture, since they had largely lost the T6 antigen. In neither of these two cases, however, had maturation progressed toward the T3-positive mature thymocyte differentiation stage. Normal T cell colonies (Table 3) contained mature activated T lymphocytes with a blastic appearance and a mature T cell immunophenotype, ie, T3+, T4 or T8+, T6−, WT1+, and E+. Colony cells of patient 6 expressed the normal phenotype. It was identical to that of the preculture cells, ie, resembling that of mature activated normal helper T cells (T3+, T4+, T6−, T8−, T10+). The fact that these colonies were indeed leukemic was apparent from cytogenetic analysis. It was found that 91% of the colony cells in metaphasis had the complex cytogenetic abnormality [46, XY, 6q−, 7p+, t(8p+; 11p−q?), 12 q+, −14, 15p+, + Mar (14q−?)] that had been demonstrated in 76% of the fresh NHL cells in metaphasis.

Table 3. Surface Membrane Antigens on Colony Cells

	Percentage of Positive Colony Cells†					
Patient*	WT1	T3	T4	T6	T8	E
1	96	0	0	5	1	0
2	77	1	13	0	0	95
3	88	3	0	3	0	85
4	86	0	0	0	98	90
5	65	4	70	16	9	73
6	100	58	85	0	0	96
Normal T	65	90	60	0	26	86
Normal T	88	87	67	0	27	90

*Preculture phenotypes of the leukemic cells are given in Table 1.

†Assessed by indirect immunofluorescence (200 cells counted)

Analysis of growth requirements of leukemic T cells that do not depend on the addition of IL 2 for proliferation. Colony formation in three cases occurred in spite of the absence of exogenous IL 2 (ie, in patients 3 and 5 with TPA alone and in patient 6 with PHA alone) (Table 2, Fig 2). Possible explanations for these findings are that these leukemias either proliferated independently on IL 2 or that IL 2 was needed but provided by the neoplastic cells themselves. To clarify this question, the cells of patient 6 (NHL) were studied in TdR incorporation microculture assays (Fig 3). First, it was investigated whether the cells were capable of IL 2 production. Culture supernatants conditioned by PHA-activated T-NHL cells (T-NHL–CM) were tested for IL 2 activity by inducing proliferation of CTLL cells (murine IL 2–dependent cell line). From Fig 3A it can be seen that increasing concentrations of T-NHL–CM stimulated CTLL proliferation progressively. IL 2 activity of the T-NHL–CM was estimated at approximately 5 u/mL by comparison with dose response relationships of pure r–IL 2. Without PHA

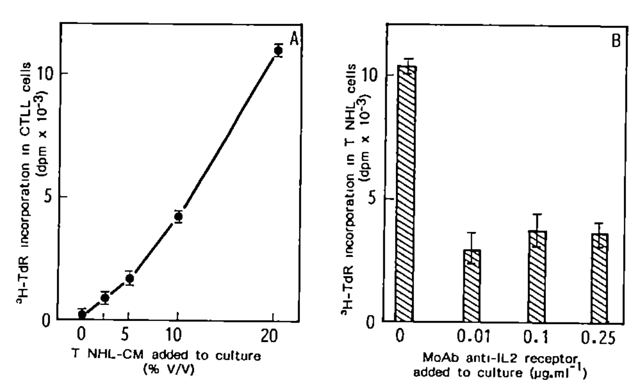

Fig 3. Microculture analysis of IL 2 production (A) and the effect of IL 2 receptor blocking on proliferation (B) (T-NHL patient 6). A. Dose-dependent proliferation induction of fully IL 2–dependent murine CTLL cells by increasing concentrations of culture medium conditioned by the leukemic T cells (T-NHL–CM). B. Inhibition of proliferation of T-NHL cells in cultures with PHA and without exogenous IL 2 following the addition of MoAb aIL 2r at three different concentrations.

activation, the culture media conditioned by the NHL cells contained only 1 U/mL IL 2 activity.

The elaboration of IL 2 in the culture medium by the NHL cells of patient 6 and the appearance of IL 2 receptors on these cells following PHA stimulation suggested that their proliferation depended on the release of endogenous IL 2. Therefore, we investigated the effect of blocking IL 2 membrane receptors (with the anti–IL 2 receptor MoAb aIL 2r) on the proliferation of the T-NHL cells in the absence of exogenous IL 2 (Fig 2B). Culturing of the cells in the presence of MoAb aIL 2r resulted in a 60% to 70% inhibition of TdR incorporation. On the other hand, (1) the incubation of T-NHL cells with control MoAb OKT3 (IgG2a) or B13.9 (IgG1) or (2) the incubation of control (acute myelogenous leukemia) cells with MoAb aIL 2r did not result in a significant suppression of TdR incorporation. Taken together, these data confirm that the T-NHL cells of patient 6 indeed require IL 2 for proliferation. Notably, however, exogenous IL 2 is not required because the cells elaborate their own IL 2 at effective concentrations.

DISCUSSION

The induction of proliferation of normal T lymphocytes in vitro involves a dual-step mechanism in which an activator (eg, antigen, lectin, phorbol ester) and the lymphokine IL 2 (previously designated as T cell growth factor) are both essential components.[18] Activation is required to induce receptors for IL 2 on the T cell membrane, thereby rendering the cells sensitive to the growth factor. The discovery that certain (HTLV-infected) leukemias of mature T cell type continuously express IL 2 receptors without prior activation[8] and the finding that some cutaneous T cell leukemia/lymphoma cell lines produce and respond to IL 2[19,20] supported the idea that the growth of leukemic T cells depends on a permanent responsiveness to IL 2 in association with the ability to self-produce this growth factor. Such a model of autostimulation driven by IL 2 was initially thought to be crucial in the development of HTLV-associated neoplasms.[19] However, this suggestion was later considered less likely because it was found that not all HTLV-induced leukemias are capable of IL 2 production. In addition, the cells of the latter neoplasms do not always require (endogenous or exogenous) IL 2 for proliferation.[21]

In another class of mature T cell leukemia, ie, malignant cutaneous T cell lymphoma (Sézary syndrome), the cells were found to produce and respond to IL 2 in vitro following stimulation with PHA and TPA.[22] Sézary cells, with few exceptions, are not infected with HTLV.[23] They generally lack spontaneous IL 2 receptor expression and are therefore dependent on mitogenic stimulation. The autocrine secretion of IL 2 by Sézary cells, however, is not a tumor-associated characteristic because normal T helper lymphocytes express the same ability.[24,25]

The role of IL 2 in the regulation of the growth of T-ALL and T-NHL has as yet remained a rather unexplored area. The present study was undertaken to establish the in vitro culture requirements of these T cell neoplasms. The results from colony culture and TdR incorporation experiments indicate that T-ALL and T-NHL cells from six individual patients had remained dependent on IL 2 (either added to the cultures or endogenously produced) for proliferation (Table 2, Figs 2 and 3). Besides IL 2, T-ALL and T-NHL cells require activation by TPA for colony growth. In all cases, colonies were formed in TPA- plus IL 2–supplemented cultures (Table 2). TPA is needed for the induction of IL 2 surface membrane receptors on the T-ALL and T-NHL cells. Fresh leukemic T cells lacked these receptors, whereas exposure to the phorbol ester resulted in their appearance on a high proportion of the cells (Table 2). The induction of receptors for IL 2 by TPA on certain leukemic T cell lines initially lacking such receptors has also been reported.[26] Thus, T-ALL and T-NHL cells share with normal T lymphocytes the requirement of activation before they are capable of responding to IL 2.

Although it is clear that the interaction of foreign antigen with antigen receptors on the cell surface is the in vivo activation signal for T lymphocytes and stage III thymocytes, no in vivo mechanism underlying activation of immature T cell types (ie, not expressing the T3 antigen receptor complex on the cell membrane) is known. Therefore, no conclusions can as yet be drawn from our in vitro experiments as regards the role of IL 2 in the proliferation of stage I and stage II thymocyte like T-ALL and T-NHL cells in vivo. However, it was recently shown that IL 2 receptors are present on a population of freshly isolated murine thymocytes with an immature (Ly2−, L3T4−) immunophenotype,[27,28] and on this basis, it was suggested that thymocytes can be activated by a stimulus intrinsic to the thymus.[27] If this hypothesis is correct, it raises the possibility that, in vivo, the IL 2 responsivenes of leukemic T cells with stage I or stage II thymocyte immunophenotypes can be induced in situ in the thymus. Notably, thymic involvement is frequently observed in T-ALL and T-NHL.

Unlike TPA, PHA exerted an effect only in the one case of T-NHL expressing a mature thymocyte (stage III) immunophenotype (Table 2, patient 6). The expression of IL 2 receptors and colony formation were induced by PHA (as well as by TPA) in this mature (stage III thymocyte) positive NHL. The other, prothymocytelike or immature (stage I, stage II) thymocytelike tumors lacked PHA responsiveness. They share this inability with their putative normal thymic counterparts.[29] With Jurkat cells and a variety of mutants derived from this cell line it was recently shown that the PHA responsiveness of T cells is directly related to the expression of the T3 antigen receptor complex on the cell surface.[30]

In three cases (ie, patients 1, 2, and 4), colony formation was strictly dependent on the addition of IL 2 to culture. The fact that IL 2 had to be added suggests that the neoplastic cells were not capable of IL 2 production. However, on the basis of our experiments we cannot exclude the possibility that some IL 2, insufficient for autostimulation, was actually released by the cells of these patients. On the other hand, in the three other cases (ie, patients 3, 5, and 6) colony growth was apparent with TPA or PHA as a single additive to culture and did not require exogenous IL 2. It was documented in one of these cases (patient 6) that the neoplastic cells produced considerable amounts of IL 2. Culture media conditioned by the T-NHL cells of patient 6 contained IL 2 activity on the order of 5 U/mL. Thus, we propose that T-ALL and T-NHL may be subdivided into categories on the basis of in vitro proliferation characteristics, ie, (1) responsive to both TPA and PHA, (2) responsive to TPA but not to PHA, (3) capable of IL 2 production in sufficient amounts for self-stimulation in colony culture, and (4) not capable of significant IL 2 production.

Normal T cells at different stages of maturation may express identical features. Therefore, the results of our in vitro studies as yet do not provide indications for a disordered response to IL 2 underlying the uncontrolled proliferation in T-ALL and T-NHL. However, it is at present not known whether critical abnormalities exist in these T cell leukemias as far as the detailed requirements of activation of IL 2 synthesis, activation and down-regulation of IL 2 receptors, and function of these receptors are concerned. Such abnormalities have been described for adult T cell leukemia and certain leukemic T cell lines.[6,31]

Results from immunophenotyping provided no positive evidence for differentiation of the T-ALL and T-NHL cells during colony formation in cultures supplemented with TPA. No acquisition of new differentiation antigens (eg, E, T3, T6) indicative of in vitro maturation of the leukemic T cells was apparent (Table 1, Table 3). These data contrast with those of experiments with certain leukemic T cell lines (Jurkat, CEM, and Molt-3) in which TPA was found to induce the expression of E rosette receptors and T3 antigens.[32–34]

Cloning efficiencies of T-ALL/T-NHL cells in IL 2 plus mitogen-containing cultures were significantly lower than those of normal peripheral T cells (Table 2). This raises the question as to whether culture conditions were suboptimal for T-ALL and T-NHL, for example, because T-ALL and T-NHL cells require other lymphokines for proliferation besides IL 2. The finding that the aforementioned subpopulation of mouse thymocytes (ie, early Ly2−/L3T4− thymocytes) do not respond in vitro to IL 2, although the cells express functional (ie, high affinity) IL 2 membrane receptors,[27,28] suggests that other growth factors in combination with IL 2 act on early stages of (murine) T cell maturation and possibly on the leukemic counterparts of the analogous cell types.

Results from previous work on non-T–ALL indicated that in vitro colony formation by common and pre-B–ALL cells depends on the combined exposure to IL 2 and a second factor elaborated by feeder leukocytes [16] However, we could not demonstrate a significant effect of the same crude source of stimulation on colony-forming cells of ALL/NHL of T lineage (results not shown). To further elucidate the critical requirements additional to IL 2 for in vitro colony growth of T-ALL and T-NHL cells, the availability of pure (recombinant) growth factor preparations will be essential.

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REFERENCES

1. Sporn MB, Todaro GJ: Autocrine secretion and malignant transformation of cells. N Engl J Med 303:878, 1980

2. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin CH, Huang JS, Deuel TF: Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304 35, 1983

3 Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD: Close similarity of epidermal growth factor receptor and *v-erb*-B oncogene protein sequences Nature 307 521, 1984

4. Sporn MB, Roberts AB: Autocrine growth factors and cancer. Nature 313:745, 1985

5. Devos R, Plaetinck G, Cheroutre H, Simons G, Degrave W, Taveriner J, Remant E, Fiers W Molecular cloning of human interleukin 2 cDNA and its expression in *E coli* Nucleic Acids Res 11·4307, 1983

6. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (antiTac) reactive with activated and functionally mature human T cells J Immunol 126:1393, 1981

7. Wong-Staal F, Gallo RC: The family of human T-lymphotropic leukemia viruses: HTLV-I as the cause of adult T cell leukemia and HTLV-III as the cause of acquired immunodeficiency syndrome. Blood 65:253, 1985

8. Greaves MF, Rao J, Hariri G, Verbi W, Catovsky D, Kung P, Goldstein G: Phenotypic heterogeneity and cellular origins of T cell malignancies. Leuk Res 5:281, 1981

9 Nadler LM, Stashenko P, Reinherz E, Ritz J, Hardy R, Schlossman SF: Expression of normal differentiation antigens on human leukemia and lymphoma cells, in Rosenberg SA, Kaplan HS (eds): Malignant Lymphomas. Etiology, Immunology, Pathology, Treatment Cancer Symposia, vol 3. New York, Bristol Myers, 1982, p 107

10. Böyum A Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 21:77, 1968 (suppl 97)

11. Madsen M, Johnsen HE, Wendelboe Hansen P, Christiansen SE: Isolation of human T and B lymphocytes by E-rosette gradient centrifugation. Characterization of the isolated subpopulations. J Immunol Methods 33.323, 1980

12. Touw I, Löwenberg B Variable differentiation of human acute myeloid leukemia during colony formation in vitro· A membrane marker analysis with monoclonal antibodies. Br J Haematol 59:37, 1985

13 Tax WJM, Tidman N, Janossy G, Trejdosiewicz L, Willems R, Leeuwenberg J, de Witte TJM, Capel PJA, Koene RAP Monoclonal antibody (WTl) directed against a T cell surface glycoprotein. Characteristics and immunosuppressive activity. Clin Exp Immunol 55.427, 1984

14. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC: A monoclonal antibody that appears to recognize the receptor for human T cell growth factor. Partial characterization of the receptor. Nature 300.267, 1982

15. Löwenberg B, de Zeeuw MC: A method for cloning T lymphocytic precursors in agar. Am J Hematol 6:35, 1979

16. Touw I, Delwel R, Bolhuis R, van Zanen G, Löwenberg B: Common and pre-B acute lymphoblastic leukemia cells express

interleukin 2 receptors and interleukin 2 stimulates in vitro colony formation. Blood 66:556 1985

17. Rosenstreich DL, Mizel, SB. Signal requirements for T lymphocyte activation I. Replacement of macrophage function with phorbol myristic acetate. J Immunol 123.1749, 1979

18 Morgan DA, Ruscetti FW, Gallo RC: Selective in vitro growth of T lymphocytes from normal human bone marrows. Science 193.1007, 1976

19. Gallo RC, Wong-Staal F: Retroviruses as etiologic agents of some animal and human leukemias and lymphomas and as tools for elucidating the molecular mechanism of leukemogenesis. Blood 60:545, 1982

20. Gootenberg JE, Ruscetti FW, Mier JW, Gazdar A, Gallo RC: Human cutaneous T cell lymphoma and leukemia cell lines produce and respond to T cell growth factor. J Exp Med 154.1403, 1981

21. Arya SK, Wong-Staal F, Gallo RC: T-cell growth factor· Lack of expression in human T-cell leukemia virus infected cells. Science 223.1086, 1984

22. Solbach W, Lange CE, Röllinghof M, Wagner H: Growth, interleukin 2 production, and responsiveness to IL 2 in T4-positive T lymphocyte populations from malignant cutaneous T cell lymphoma (Sézary's syndrome): The effect of cyclosporine A Blood 64:1022, 1984

23. Waldman TA, Greene WC, Sarin PS, Saxinger C, Bladney DW, Blattner WA, Goldman CK, Bongiovanni K, Sharrow S, Depper JM, Leonard W, Uchiyama T, Gallo RC: Functional and phenotypic comparison of human T cell leukemia/lymphoma virus positive adult T cell leukemia with human T cell leukemia/lymphoma virus negative Sézary leukemia, and their distinction using anti-Tac monoclonal antibody identifying the human receptor for T cell growth factor. J Clin Invest 73:1711, 1984

24 Wee SL, Bach FW: Functionally distinct human T cell clones that produce lymphokines with IL2-like activity. Hum Immunol 9.175, 1984

25 Meuer SC, Hussey RE, Cantrell DA, Hodgdon JC, Schlossman SF, Smith KA, Reinherz EL: Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2–dependent autocrine pathway. Proc Natl Acad Sci USA 81·1509, 1984

26. Greene WC, Robb RJ, Depper JM, Leonard WJ, Drogula C, Svetlik PB, Wong-Staal F, Gallo RC, Waldmann TA: Phorbol diester induces expression of Tac antigen on human acute T lymphocytic leukemic cells. J Immunol 133.1042, 1984

27. Ceredig R, Lowenthal JW, Nabholz M, MacDonald HR: Expression of interleukin-2 receptors as a differentiation marker on intrathymic stem cells. Nature 314:98, 1985

28. von Boehmer H, Crisanti A, Kisielow P, Haas W: Absence of growth by most receptor-expressing fetal thymocytes in the presence of interleukin-2. Nature 314:539, 1985

29. De Vries JE, Vyth-Dreese FA, Figdor CG, Spits H, Leemans JM, Bont WS: Induction of phenotypic differentiation, interleukin 2 production and PHA-responsiveness of "immature" human thymocytes by interleukin 1 and phorbol ester. J Immunol 131.201, 1983

30. Weiss A, Stobo JD: Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line J Exp Med 160:1284, 1984

31. Tsudo M, Uchiyama T, Uchino H, Yodoi J: Failure of regulation of Tac antigen/TCGF receptor on adult T-cell leukemia cells by antiTac monoclonal antibody Blood 61:1014, 1983

32. Nagasawa K, Mak TW: Phorbol esters induce differentiation in human malignant T lymphoblasts. Proc Natl Acad Sci USA 77:2964, 1980

33. Nagasawa K, Mak TW: Induction of human malignant T-lymphoblastic cell lines Molt-3 and Jurkat by 12-0-tetradecanoyl-phorbol-13-acetate: Biochemical, physical and morphological characterization. J Cell Physiol 109:181, 1981

34. Ryffel B, Henning CB, Huberman E: Differentiation of human T-lymphoid leukemia cells into cells that have a suppressor phenotype is induced by phorbol 12-myristate 13-acetate. Proc Natl Acad Sci USA 79:7336, 1982

Large Granular Lymphocytes From Patients With Expanded LGL Populations Acquire Cytotoxic Functions and Release Lymphokines Upon In Vitro Activation

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The phenotypic and functional features of purified large granular lymphocytes (LGL) from ten patients with LGL population expansions and cytopenias are described. The predominant LGL phenotypes were T3+, T8+, Leu-11+/−; however, in two patients, LGL expressed a T3−, Leu-11+ phenotype. Variable combinations of other LGL markers (OKM1, Leu-7), and HLA-DR were detected in individual cases. In nine of ten cases, freshly isolated LGL did not exert cytolytic activity for K562 target cells, but purified LGL cultured in the presence of recombinant interleukin 2 (rIL2) acquired potent cytotoxic activity in all cases tested. LGL did not proliferate in response to phytohemagglutinin (PHA). However, LGL released variable amounts of IL2 and γ-interferon (γ-IFN) after PHA stimulation. In some cases, stimulation of fresh LGL with recombinant IL2 induced production of γ-IFN. No correlation was found between the functional capabilities and the original phenotype of the expanded LGL populations.

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LARGE GRANULAR LYMPHOCYTES (LGL) are a subset of lymphoid cells defined by morphologic and cytochemical criteria.[1-3] These cells display abundant cytoplasmic azurophilic granules that stain with the cytochemical reactions for lysosomal acid hydrolases but are peroxidase-negative.[2,3]

Several techniques for LGL purification have recently been developed[4-6] that have allowed surface phenotype analysis and determination of some functional properties of these cells.[7] It has been shown that LGL are phenotypically heterogeneous, the majority of them being OKMI$^+$, Leu-11+, Leu-7+, and T3−, T8−. Other cells express T3 and T8 determinants and are Leu-11−.[5,6,8] Small LGL subsets may express various combinations of these markers. The majority of the LGL mediate cytotoxic functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and natural killer (NK) cell activity.[1,4,7] Other LGL are poorly cytolytic, but regulate B cell proliferation.[9] Besides these two functionally distinct LGL subsets, other cells are capable of regulating cell proliferation and differentiation and have cytotoxic functions as well.[10-18]

Numerous studies have described patients with expanded LGL populations.[19-21] The surface phenotype of the LGL varied in the different patients, but within each single case, cells displayed a high degree of phenotypic homogeneity. The availability of these homogeneous LGL populations provides the opportunity to investigate the relationship between surface markers and cell function. Assessment of the properties of expanded LGL populations may, in turn, contribute to understanding the origin of LGL in these patients as well as in normal individuals.

In this study we have analyzed the surface phenotype, the cytotoxic ability, and the cytokines released by purified LGL from ten patients with abnormally expanded LGL populations of homogeneous phenotype.

MATERIALS AND METHODS

Patients

Patients studied had not received treatment with steroids or cytotoxic drugs for at least 5 weeks. Two patients (A.F. and J.G.) were asymptomatic Two patients (F.H and V.F) had transfusion-dependent anemia only, and four (M.P., K.M., A.S., B.F.) had recurrent neutropenia and infections. Of these, one (K.M.) had immune neutropenia in remission at the time of the study, and one had anemia and thrombocytopenia (M.P.). One (S.W.) had seropositive rheumatoid arthritis, and another (P.B.) had anemia and posttransfusion purpura. All patients had peripheral blood and bone marrow lymphocytosis at some time in the clinical course and persistent or recurrent expansion of LGL with a homogeneous surface phenotype defined by multiple-cell surface marker determinations For the clinical definition of LGL expansions see Newland et al[20] and Landay et al [21] On physical examination three patients (K.M., P.B , and S.W.) had splenomegaly, but none had skin lesions, lymphadenopathy, or hepatomegaly. All patients remain alive and continue to have persistent elevations of LGL with a consistently stable phenotype. Laboratory features of the patients are summarized in Table 1.

Cell Separation Procedures

Heparinized venous blood samples were obtained from patients after informed consent. Mononuclear cells (MNC) were separated on Ficoll-Hypaque (F-H) density gradients and partially depleted of monocytes by plastic adherence. Cells forming rosettes with neuraminidase-treated sheep erythrocytes were isolated by incubation of adherent cell–depleted MNC with indicator cells, followed by fractionation on two subsequent F-H density gradients as previously reported.[17] Sheep erythrocytes were removed from cells forming E rosettes by hypotonic lysis.

E+ cells highly enriched for LGL, as determined by morphologic and cytochemical criteria to be detailed, were treated with a monoclonal antibody with specificity for the helper-inducer T cell subset, (OKT4, Ortho Pharmaceutical Corp, Raritan, NJ), a monocyte-specific reagent (Leu-MI, Becton Dickinson, Sunnyvale, Calif), and complement. In two cases with T8- LGL expansion, the OKT8 monoclonal antibody (Ortho) that reacts with cytotoxic-suppressor T cells was included in the cytotoxicity assay. Cells were suspended

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at a concentration of 1.5 × 10^7/mL in RPMI 1640 medium containing OKT4 (at a final dilution of 1:50), Leu M1 (at a final dilution of 1:20), and OKT8 (at a final dilution of 1:50). After one hour incubation on ice, nontoxic rabbit complement (Pel-Freeze, Rogers, Ariz) was added at a final dilution of 1:2 in RPMI 1640. Cells were incubated at 37 °C for one hour, washed twice, resuspended in fresh complement, and incubated at room temperature for an additional 30 minutes. Nonviable cells were removed by centrifugation on F-H density gradients. To assess the efficiency of antibody and complement-mediated cytolysis, the proportion of nonviable cells was determined by trypan blue or ethidium bromide dye exclusion and compared with that of immunofluorescence staining using the same antibody. By immunofluorescence staining it was determined that complement-mediated lysis killed at least 80% of the cells.

Cell Surface Marker Analysis

B cells were identified by staining with the B1 monoclonal antibody (Coulter Immunology, Hialeah, Fla). Anti–T cell reagents (Leu-2, Leu-3, Leu-4, Leu-9) and the antisheep red cell receptor monoclonal antibody Leu-5 were obtained from Becton Dickinson. Anti–interleukin 2 (IL2) receptor monoclonal antibodies were kindly donated by Dr T Waldmann (anti-Tac)[22] and Dr A. Moretta (H-108). Anti–HLA-DR antibodies (HLA-DR or PTF 29/12) were obtained from Becton Dickinson and from Dr G. Damiani,[23] respectively. OKM1, a monocyte/macrophage-specific monoclonal antibody that also stains NK cells, was obtained from Ortho. A monocyte/macrophage-specific monoclonal antibody, Leu-M1, was purchased from Becton Dickinson, as well as two LGL-specific monoclonal antibodies, Leu-7 and Leu-11b.

All of the aforementioned reagents were used in indirect immunofluorescence tests with fluorescein isothiocyanate (FITC)-conjugated goat antimouse immunoglobulin (Southern Biotechnology Associates, Birmingham, Ala)

Flow Cytometry Analysis

Cell preparations stained for immunofluorescence were fixed with 1% paraformaldehyde and analyzed by flow cytometry using a FACS Analyzer (Beckton Dickinson). Ten thousand cells were run through appropriately set gates Control preparations consisted of unstained cells, cells stained with the labeled second reagent alone, and cells stained with unrelated antibodies of the same isotype as the test antibodies. In some experiments, wet cell preparations were also analyzed with a Leitz Orthoplan fluorescence microscope. The percentage of positive cells was determined by enumeration of at least 200 cells

Cytochemistry and Criteria for LGL Identification

LGL were identified by Wright staining of cytocentrifuged cell preparations or by cytochemical localization of alpha naphthyl acetate esterase (ANAE), acid phosphatase (AP), or β-glucuronidase (β-gluc) as previously reported.[3]

Cell Culture

One × 10^6 purified LGL were cultured for 48 hours in 24-well plates (Costar, Cambridge, Mass), in RPMI 1640 supplemented with 10% fetal calf serum (RPMI-FCS), with or without 1 μl/mL phytohemagglutinin (PHA, Difco, Detroit). In some experiments, 1 × 10^6/mL LGL were cultured for 48 hours in RPMI-FCS supplemented with 20 IU/mL recombinant IL2 (rIL2) (Biogen, Lausanne, Switzerland), the biologic activity of which had been titrated as previously reported.[24] Cultures were harvested, and supernatants were recovered and frozen at −70 °C until testing. To determine cell proliferation, purified LGL (5 × 10^5/mL) were suspended in RPMI-FCS and cultured in triplicate in 96-well microplates (Costar) (1 × 10^5 cells/well) for 48 hours with or without 1 μL/mL PHA. Cultures were subsequently pulsed with 1 μCi/well ^{3}H-thymidine 12 hours before harvesting Radioactivity was determined by liquid scintillation counting and results expressed as cpm/min (mean ± SE of triplicate cultures)

Cytotoxicity Assays

NK cell activity and rIL2–activated killing (AK) were assayed in triplicate in a four-hour ^{51}Cr release test as previously reported.[24] The target cell used for both assays was the erythroleukemia cell line K562. Effector cells were (1) freshly purified LGL for the NK assays and (2) LGL cultured for three days with 20 IU/mL rIL2, for the AK assay The SD of percent specific ^{51}Cr release was less than 20% in all experiments

Lymphokine Assays

Colony-stimulating factor (CSF). Normal peripheral blood MNC depleted of adherent cells and of cells forming E rosettes were used as targets. Two × 10^5 cells were resuspended in a final volume of 1 mL of a mixture containing α-medium (GIBCO, Grand Island, NY), 10% heat-inactivated FCS, 0.8% methylcellulose (Dow Chemical Co, Midland, Mich), and supplemented with the nonessential amino acids L-glutamine and sodium pyruvate [25] Supernatants to be tested were included in the culture medium at the final concentration of 10% Control plates contained 10% conditioned medium of the GCT line (GIBCO) as a source of CSF. Cultures were incubated at 37 °C in an atmosphere containing 5% CO_2 in air and scored for

Table 1. Hematologic Features of Patients with Expanded LGL Populations*

Patient	WBC/μL	Percentage of Lymphocytes	Percentage of Granulocytes	Absolute Lymphocyte Counts/μL	Absolute Granulocyte Counts/μL
A F.	22,900	93	6	21,297	1,374
M.P.	2,400	48	46	1,152	1,104
F H.	27,500	89	8	24,475	2,200
V.F.	8,000	65	21	5,200	1,680
J.G.	10,300	66	24	6,798	2,472
K.M	4,100	55	29	2,255	1,189
P B	10,800	69	29	7,452	3,132
S W.	6,600	63	25	4,158	1,650
A.S.	1,500	80	15	1,200	225
B.F	10,500	90	8	9,450	840

*Features are determined in peripheral blood at the time of LGL purification.

Table 2. Phenotypic Characteristics of Peripheral Blood MNC From Patients With Expanded LGL Populations

	Surface Markers*												
Patient	Leu-9	Leu-5	Leu-4	Leu-3	Leu-2	OKM1	Leu-7	Leu-11	Tac	HLA-DR	B1	Leu-M1	Percentage of LGL†
A F	71 (91)	95 (98)	98 (97)	6 (<1)	2 (<1)	95 (96)	89 (92)	95 (96)	<1 (<1)	10 (3)	2 (<1)	2 (<1)	95 (97)
M P	68 (78)	87 (98)	82 (97)	31 (<1)	53 (96)	31 (45)	42 (54)	20 (31)	<1 (<1)	42 (35)	4 (<1)	5 (<1)	45 (95)
F H	90 (96)	98 (99)	96 (98)	2 (<1)	97 (96)	34 (45)	96 (97)	95 (97)	<1 (<1)	6 (1)	1 (<1)	1 (<1)	98 (98)
V F	92 (98)	95 (99)	93 (98)	16 (<1)	78 (85)	55 (60)	62 (70)	76 (84)	<1 (<1)	68 (65)	1 (<1)	2 (<1)	65 (98)
J G	84 (88)	97 (99)	5 (<1)	5 (<1)	6 (<1)	82 (85)	19 (25)	87 (90)	<1 (<1)	81 (85)	<1 (<1)	2 (<1)	98 (99)
K M	86 (91)	89 (97)	12 (<1)	15 (<1)	3 (<1)	75 (90)	25 (39)	59 (75)	<1 (<1)	2 (<1)	<1 (<1)	1 (<1)	60 (96)
P B	57 (66)	80 (97)	57 (95)	17 (<1)	36 (53)	48 (63)	47 (65)	29 (35)	<1 (<1)	20 (<1)	20 (<1)	4 (<1)	45 (97)
S W	66 (75)	87 (99)	88 (96)	6 (<1)	83 (91)	88 (95)	3 (5)	85 (92)	<1 (<1)	67 (76)	<1 (<1)	3 (<1)	70 (96)
A S	85 (92)	80 (97)	75 (95)	8 (<1)	68 (77)	7 (5)	65 (79)	50 (65)	<1 (<1)	8 (<1)	2 (<1)	3 (<1)	60 (95)
B F	90 (96)	92 (98)	90 (97)	5 (<1)	82 (91)	2 (<1)	75 (83)	65 (76)	<1 (<1)	5 (<1)	1 (<1)	4 (<1)	70 (98)

*As determined by immunofluorescence microscopy or fluorescence-activated cell sorter analysis Data are the percentage of positive cells Data in parentheses refer to the percentage of positive cells determined for purified LGL preparations

†As determined by Wright's staining and cytochemical staining for AP and β-gluc

colonies after 14 days. Colonies were defined as aggregates containing more than 50 cells.

IL2 assay IL2 activity was measured by determining the ability of the IL2–dependent murine cell line CTLL-2 to proliferate.[26] CTLL-2 cells were washed twice, resuspended in RPMI-FCS, and cultured for 24 hours in 96-well, flat-bottom plates at the concentration of 1×10^4/well in the presence or absence of various dilutions of the supernatants to be tested. Cells were pulsed with 1 μCi^{3}H-thymidine six hours before harvesting, and radioactivity was determined by liquid scintillation counting. IL2 units were calculated by probit analysis[26] using r-IL2 (Biogen) as a reference.

γ-Interferon (γ-IFN) assay γ-IFN was measured by a radioimmunoassay (IMRX, Centocor Inc, Malvern, Pa) based upon the use of (1) polystyrene beads coated with a mouse monoclonal antibody specific for human γ-IFN that are incubated with test supernatants and subsequently washed and (2) a second monoclonal antibody to human γ-IFN labeled with ^{125}I that reacts with γ-IFN bound to the beads. The radioactivity bound to the beads, quantified in a gamma-scintillation counter, is proportional to the concentration of γ-IFN in the specimen, within the working range of the assay. Results were expressed in units calculated using a preparation of recombinant γ-IFN as standard reference.

RESULTS

Characterization of Abnormally Expanded LGL Populations

The surface phenotype of peripheral blood MNC from ten patients with expanded LGL populations is shown in Table 2. In all cases, LGL were Leu-5+ and Leu-9+ and most expressed the Leu-4, Leu-2, Leu-7, OKM1, and Leu-11 antigens. HLA-DR molecules were expressed on LGL from four out of ten patients. LGL from all cases were negative for Leu-3 and Leu-M1 antigens. In agreement with previous observations on expanded LGL populations,[27] LGL stained for AP and β-gluc with a paranuclear localization of the enzyme activity, but were weakly reactive or negative for ANAE. Percentages of LGL were quantified in peripheral blood MNC fractions by morphologic criteria and by cytochemical staining for AP and β-gluc (Table 2.)

Lymphokine Production by Purified LGL

Because all of the expanded LGL populations expressed receptors for sheep erythrocytes, LGL enrichment was achieved by E rosetting. T cells with a helper phenotype and monocytes were removed from the cell preparations by treatment with OKT4 and Leu-M1 monoclonal antibodies and complement. In the two cases in which LGL did not express the T8 antigen, cells forming E rosettes were also treated with OKT8 monoclonal antibody and complement. As apparent, purified LGL had surface phenotype and cytochemical staining patterns that were highly homogeneous in all of the cases (Table 2).

Purified LGL were cultured in vitro in the presence and absence of PHA, and cell proliferation was measured by ^{3}H-thymidine uptake at 48 hours. Spontaneous proliferation was never detected. With one exception (patient S.W.), no ^{3}H-thymidine incorporation above background values was observed in PHA-stimulated cultures. Addition of allogeneic monocytes did not increase responsiveness to PHA (data not shown).

Supernatants of unstimulated and of PHA-stimulated LGL cultured for two days were tested for their IL2 and γ-IFN content (Table 3). Most of the LGL preparations produced >2 U/mL of IL2 with PHA stimulation only. In unstimulated culture supernatants from three patients

Table 3. γ-IFN and IL 2 Production by Stimulated LGL From Patients With Abnormal LGL Expansions

	Unstimulated*†		PHA Stimulation*†		rIL 2 Stimulation†‡
Patient	γ-IFN	IL 2	γ-IFN	IL 2	γ-IFN
A.F.	0	2.0	0	2.5	ND
M.P.	0	2.0	6.6	7.5	ND
F.H.	0	2.1	0	3.7	0
V.F.	0	3.2	2.7	14.0	0
J G	7 5	2.2	86	10.4	84
K.M.	0	1.2	70	9.7	ND
P B.	0	4 9	55	8.3	72
S.W.	0	6.8	84	23 0	5.3
A.S.	0	1.3	15	1.3	ND
B.F.	0	2.2	28	11 5	ND

Abbreviation: ND, not determined

*Purified LGL were cultured for 48 hours in the presence or absence of PHA. Supernatants were removed and tested

†See the Materials and Methods section for γ-IFN and IL 2 assays.

‡Purified LGL were cultured for 48 hours with 20 IU/mL rIL 2. Supernatants were removed and tested.

All values are units per milliliter

(W.F., P.B., and S.W.), >2 U/mL of IL2 were also detected. In seven of ten cases, LGL released γ-IFN following PHA stimulation (Table 3). Spontaneous γ-IFN production was observed in one case only (J.G.).[28] A good correlation between IL2 and γ-IFN production was detected in individual cases, ie, those LGL preparations that released the highest amounts of IL2 were also the most efficient γ-IFN producers. Conversely, poor IL2 producers did not release γ-IFN (Table 3).

LGL from five patients were cultured for 48 hours with rIL2, and the supernatants were tested for γ-IFN (Table 3). In two cases, IL2 stimulation induced production of high amounts of γ-IFN. In four out of five cases, a good correlation was observed between the amount of γ-IFN released with PHA stimulation and that produced in response to IL2 stimulation (Table 3).

Supernatants from PHA- and rIL2–stimulated LGL were also tested for the presence of CSF, but no CSF production was detected in any of the LGL supernatants. In contrast, control cultures with GCT-conditioned medium yielded 25 to 35 colonies/2 × 10^5 cells.

Natural and rIL2–Activated Cytotoxicity of Purified LGL

The NK activity of purified LGL was assayed against K562 cells in a four-hour ^{51}Cr release assay. Freshly purified LGL from nine patients did not exert NK activity (Table 4), but the cells from patient J.G. displayed a potent cytotoxic ability.

LGL suspensions from eight patients were also cultured for three days with rIL2 and subsequently tested for their cytolytic activity against K562 cells. Incubation with rIL2 induced a potent AK activity in all of the cell preparations (Table 4). In the five cases tested, no correlation was found between γ-IFN production and the ability to lyse K562 cells following culture with rIL2 (Tables 3 and 4).

DISCUSSION

The expanded LGL populations described in this study had surface phenotypes that were different among cases, although they were homogeneous in the single patients. However, cells from most patients released the same cytokines (ie, IL2 and γ-IFN) and were inducible by rIL2 to mediate cytotoxicity regardless of the surface phenotype. These results are relevant in view of the finding that LGL from two of the cases showed chromosomal abnormalities.[29] This observation, and the homogeneity of the surface membrane and cytochemical phenotype of LGL in each individual case, suggests that there is a clonal origin of some expanded LGL populations. This hypothesis is also supported by studies on the rearrangement of genes encoding for the β-chain of the T cell receptor in several LGL expansions with a T3+, T8+ phenotype.[30–35] The possibility that contaminant cells were responsible for lymphokine production was excluded by the method of purification used and by the appropriate control of the surface phenotypes following the purification procedure. Therefore, LGL with different surface phenotypes appear to share a number of common functional properties.

Our study of homogeneous LGL preparations also provided information on the mechanisms that may regulate lymphokine release. LGL released both IL2 and γ-IFN following PHA stimulation, whereas in some cases, stimulation with rIL2 induced γ-IFN production. This suggests that LGL activation may be a stepwise process such that PHA or other stimulants may induce IL2 production, which, in turn, triggers γ-IFN release. Occurrence of this sequence of events in highly homogeneous, likely clonal, LGL populations suggests the existence of mechanisms whereby product(s) of the clone may control function of cells within the same clone. These findings are in accordance with previous data on LGL populations isolated from normal peripheral blood showing that IL2 triggers γ-IFN release.[36]

Purified LGL that were generally devoid of NK activity for K562 cells became cytotoxic against this target after a three-day incubation with rIL2. All LGL suspensions acquired a potent AK activity, whereas some of them only released γ-IFN in response to IL2, a finding that strongly supports the contention that IL2 was per se responsible for the induction of lytic activity. Alternatively, stimulation with rIL2 might promote release of other, nonidentified factors capable of inducing AK activity.

The mode of activation of LGL observed in the present study deserves further comments. First, highly purified, homogeneous LGL could be stimulated by PHA to release lymphokines, even in the apparent absence of macrophages and IL1. This finding may indicate that LGL are already activated in vivo and have therefore received the necessary signals delivered by macrophages. Alternatively, cytokine production by LGL may be IL1/macrophage–independent and, most likely, unrelated to cell division since PHA did not induce LGL proliferation in short-term culture. It is possible that LGL themselves produced IL1,[14] which, however, could not be measured in our experimental system because of the concomitant release of IL2. Second, abnormally expanded LGL populations were particularly sensitive to signals delivered by rIL2, which induced γ-IFN release and natural cytotoxicity. These findings suggest that LGL were preactivated in vivo. Alternatively, it is possible that expression of

Table 4. Natural and rIL 2–Activated Killing of Purified LGL From Patients With LGL Expansions

Patients	NK Activity*	AK Activity†
A.F.	2	44
M.P.	3	25
F.H.	5	29
V.F.	3	21
J G.	65	59
K.M	2	83
P.B.	2	68
S.W	1	34
A.S	3	ND
B F	2	ND

*Percent specific ^{51}Cr release by K562 cells. Data shown were obtained at a 10:1 effector-target ratio.

†Purified LGL were cultured with rIL 2 (20 IU/mL) for three days. Data shown are percent specific ^{51}Cr release by K562 cells at 10:1 effector-target ratio.

IL2 receptors is a constitutive property of LGL, independent of their state of activation and/or surface phenotype.

HLA-DR expression was detected on freshly isolated LGL from four out of the ten patients studied. Class II major histocompatibility complex antigen expression was not related to (1) lymphokine production (IL2 and γ-IFN), (2) cytotoxic potential from the LGL, or (3) the clinical course.

LGL from all of the patients were consistently negative for the Tac antigen as assessed by immunofluorescence using two different anti-Tac monoclonal antibodies. One possible explanation is that LGL express surface IL2 receptors at a very low concentration. Subsequent contact with IL2 could then induce IL2 receptor expression. This phenomenon has been shown for normal B cells[37] and NK cells,[38] but does not seem to occur in the case of expanded LGL populations because the latter cells fail to stain with the anti-Tac reagent following prolonged IL2 stimulation.[29]

An alternative possibility is that at the time of surface staining with monoclonal antibodies, membrane IL2 receptors were saturated with their ligand, thus preventing identification with anti-Tac reagents. Since expanded LGL populations released IL2, the LGL themselves could provide IL2 for receptor blockade. Although this possibility cannot be dismissed, it is made unlikely by the observation that LGL required the addition of exogenous IL2 for their proliferation in vitro. A final, and highly intriguing, hypothesis is that LGL may bind IL2 through a molecular structure different from the Tac antigen. Another contrasting feature of expanded LGL, compared with normal LGL,[13] is the inability of the former cells to release detectable amounts of CSF following mitogen stimulation.

The presence of shared functional properties in LGL with different surface phenotypes is relevant to the vexing problem of LGL lineage. All of the populations examined in this study expressed the Leu-9 antigen which is normally found in prethymic T cell precursors[39] and is retained throughout T cell maturation. This finding may lead to speculation that a subset of Leu-9+ precursors may acquire LGL features and mature in the bone marrow (T3− LGL), whereas other Leu-9+ cells migrate into the thymus and subsequently express the T3 molecule (T3+ LGL). In this perspective, the majority of expanded LGL populations may be viewed as cells frozen (by the neoplastic event) at different maturational stages in different patients. Another possibility is that T3− LGL represent a prethymic stage of LGL ontogeny and that T3+ LGL are postthymic cells. This hypothesis may find support in the observation of rearranged D-J gene segments of the T cell receptor in T3− cell clones with NK function and of complete rearrangements of genes encoding for the β chain of the T cell receptor in T3+ NK clones.[40]

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REFERENCES

1. Saksela E, Timonen T, Ranki A, Hayry P: Morphological and functional characterization of isolated effector cells responsible for human natural killer activity to fetal fibroblasts and to cultured cell line targets Immunol Rev 44:71, 1979

2. Ferrarini M, Cadoni A, Franzi AT, Ghigliotti C, Leprini A, Zicca A, Grossi CE. Ultrastructure and cytochemistry of human peripheral blood lymphocytes Similarities between the cells of the third population and T_G lymphocytes. Eur J Immunol 10:562, 1980

3. Grossi CE, Cadoni A, Zicca A, Leprini A, Ferrarini M: Large granular lymphocytes in human peripheral blood. Ultrastructure and cytochemical characterization of the granules. Blood 59:277, 1982

4. Timonen T, Ortaldo JR, Herberman RB: Characterization of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153:369, 1981

5. Ortaldo JR, Sharrow SO, Timonen T, Herberman RB: Determination of surface antigens on highly purified human NK cells by flow cytometry and monoclonal antibodies. J Immunol 127:2401, 1981

6 Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF: Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 131:1789, 1983

7. Ortaldo JR, Herberman RB: Heterogeneity of natural killer cells. Ann Rev Immunol 2·359·1984

8. Perussia B, Starr S, Abraham S, Fanning V, Trinchieri G. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking FcR functions. I. Characterization of the lymphocyte subset reactive with B73.1. J Immunol 150.2133, 1983

9 Pistoia V, Cozzolino F, Torcia M, Castigli E, Ferrarini M· Production of B cell growth factor by a Leu-7+, OKM1+ non-T cell with the features of large granular lymphocytes (LGL). J Immunol 134:3179, 1985

10. Hansson M, Beran M, Andersson B, Kiessling R. Inhibition of in vitro granulopoiesis by autologous and allogeneic human NK cells. J Immunol 129:126, 1982

11. Arai S, Yamamoto H, Itoh K, Kumagai K: Suppressive effect of human natural killer cells on pokeweed mitogen-induced B cell differentiation. J Immunol 131:651, 1983

12. Tilden AB, Abo T, Balch CM· Suppressor cell function of human granular lymphocytes identified by the HNK-1 (Leu-7) monoclonal antibody. J Immunol 132:611, 1984

13. Kasahara T, Djeu JY, Dougherty SF, Oppenheim JJ· Capacity of human large granular lymphocytes to produce multiple lymphokines: Interleukin-2, interferon and colony stimulating factor J Immunol 131:2379, 1983

14. Scala G, Allavena P, Djeu JY, Kasahara T, Ortaldo JR, Herberman RB, Oppenheim JJ: Human large granular lymphocytes are potent producers in interleukin-1. Nature 309 56, 1984

15 Brieva JA, Targan S, Stevens RH· NK and T cell subsets regulate antibody production by human in vivo antigen-induced lymphoblastoid B cells J Immunol 132.611, 1984

16. Mangan KF, Hartnett ME, Matis SA, Winkelstein A, Abo T· Natural killer cells suppress human erythroid stem cell proliferation in vitro. Blood 63:260, 1984

17. Pistoia V, Ghio R, Nocera A, Leprini A, Perata A, Ferrarini M: Large granular lymphocytes have a promoting activity on human peripheral blood erythroid burst forming units. Blood 65·464, 1985

18. Degli Antoni G, Perussia B, Mangoni L, Trinchieri G: Inhibition of bone marrow colony formation by human natural killer cells and by natural killer cell–derived colony inhibiting activity J Exp Med 161:1152, 1985

19. Reynolds CW, Foon KA: T-γ lymphoproliferative disease and related disorders in human and experimental animals: A review of the clinical, cellular and functional characteristics. Blood 64:1146, 1984

20. Newland AC, Catovsky D, Linch D, Cawley JC, Beverley P, San Miguel JF, Gordon-Smith EC, Blecher TE, Shahriari S, Varadi S: Chronic T cell lymphocytosis: A review of 21 cases. Br J Haematol 58:433, 1984

21. Landay A, Poon MC, Clement LT, Grossi CE: A lymphoproliferative disorder of granular lymphocytes with novel phenotype and suppressor function. J Clin Immunol 4:326, 1984

22. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac+ cells. J Immunol 126:1393, 1981

23. Corte G, Damiani G, Calabi F, Fabbi M, Bargellesi A: Analysis of HLA polymorphism by two dimensional peptide mapping. Proc Natl Acad Sci USA 78:534, 1981

24. Itoh K, Tilden AB, Kumagai K, Balch CM: Leu 11+ lymphocytes with natural killer (NK) activity are precursors of recombinant interleukin 2 (rIL-2)-induced activated killer (AK) cells. J Immunol 134:802, 1985

25. Pike BL, Robinson WA. Human bone marrow colony growth in vitro. J Cell Physiol 76:77, 1970

26. Gillis S, Ferm MM, Ou W, Smith KA: T cell growth factor: Parameters of production and quantitative microassay for activity. J Immunol 120:2027, 1978

27. Ferrarini M, Romagnani S, Montesoro E, Zicca A, Del Prete GF, Nocera A, Maggi E, Leprini A, Grossi CE: A lymphoproliferative disorder of the large granular lymphocytes with a natural killer activity. J Clin Immunol 3:30, 1983

28. Hooks JJ, Haynes BF, Detrick-Hooks B, Diehl LF, Gerrard TL, Fauci AS: Gamma (immune) interferon production by leukocytes from a patient with a T_G proliferative disease. Blood 59:198, 1982

29. Pistoia, Carroll, Prasthofer EF, Tilden AB, Ferrarini M, Zuckerman K, Grossi CE: Establishment of Tac-negative IL-2 dependent cytotoxic cell lines from large granular lymphocytes (LGL) of patients with expanded LGL populations. J Clin Immunol (in press)

30. Aisenberg A, Krontiris TG, Mak TW, Wilkes BM: Rearrangement of the gene for the beta chain of the T-cell receptor in T-cell chronic lymphocytic leukemia and related disorders. N Engl J Med 313:529, 1985

31. Bertness V, Kirsch I, Hollis G, Johnson B, Bunn PA Jr. T-cell receptor gene rearrangements as clinical markers of human T-cell lymphomas. N Engl J Med 313:534, 1985

32. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ: Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 313:776, 1985

33. O'Connor NTJ, Wainscoat JS, Weatherall DJ, Gatter KC, Feller AC, Isaacson P, Jones D, Lennert K, Pallesen G, Ramsey A, Stein H, Wright DH, Mason DY: Rearrangement of the T-cell–receptor β-chain gene in the diagnosis of lymphoproliferative disorders. Lancet 1:1295, 1985

34. Pelicci PG, Allavena P, Rambaldi A, Knowles DM, Mantovani A, Dalla-Favera R: Interleukin-2 induced T-cell receptor gene(s) rearrangements and expression in large granular lymphocytes/natural killer cells. Blood 66:159, 1985 (suppl)

35. Berliner N, Duby AD, Linch DC, Seidman JG: Monoclonal T-cell receptor gene rearrangements in T-cell lymphocytosis and cytopenia. Blood 66:96, 1985 (suppl)

36. Trinchieri G, Kobayashi M, Clark SC, Seehra J, London L, Perussia B: Response of resting human peripheral blood natural killer cells to interleukin 2. J Exp Med 160:1147, 1984

37. Waldmann TA, Goldman CK, Robb RJ, Deeper JM, Leonard WJ, Sharrow SO, Bongiovanni KF, Korsmeyer SJ, Greene WC: Expression of interleukin 2 receptors on activated human B cells. J Exp Med 160:1450, 1984

38. London L, Perussia B, Trinchieri G: Induction of proliferation in vitro of resting human natural killer cells: Expression of surface activation antigens. J Immunol 134:718, 1985

39. Vodinelich L, Tax V, Bai Y, Pegram S, Capel P, Greaves MF: A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 62:1108, 1983

40. Ritz J, Campden TJ, Schmidt RE, Royer HD, Hercend T, Hussey R, Reinherz EL: Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. Science 228:1540, 1985

Divergent Molecular Phenotypes of KG1 and KG1a Myeloid Cell Lines

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The cell line KG1 derived from a patient with erythroleukemia in myeloblastic relapse has the composite phenotype and functional repertoire of myeloblasts. In marked contrast, its subline KG1a has lost myeloid features, acquired new karyotypic markers, and has three characteristics associated with immature T cells: low-level expression of the T cell receptor β mRNA (but not α) transcribed from a germline gene; high-level expression of T3 δ mRNA and intracellular, but not cell surface, T3 protein; and expression of the CD7/gp40 T cell–associated membrane antigen. Both KG1 and KG1a transcribe unrearranged IgH genes. These data suggest that either the KG1 cell line was derived from a common myeloid-lymphoid progenitor or that the KG1a subline phenotype is aberrant.

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IN 1978 Koeffler and Golde[1] reported the establishment of a cell line, KG1, derived from a 59-year-old male patient in whom acute myeloblastic leukemia had developed following an earlier phase of erythroleukemia. This cell line had a myeloblastic phenotype and, in early passages, was colony-stimulating factor (CSF)-dependent for colony formation in soft agar and developed macrophage characteristics in response to phorbol derivatives.[1] Koeffler et al later reported[2] that a distinctive subline, KG1a, had developed after 35 passages in one of the two laboratories maintaining the KG1 cell line. The KG1a derivative had the same karyotypic abnormalities as KG1 but appeared undifferentiated and failed to express the myeloid characteristics of its parent line including cytochemical features, responses to CSF, expression of the Fcγ receptor and myeloid membrane antigen, and phagocytosis of opsonized *Candida albicans*. Koeffler suggested that KG1a was a cell line of very immature myeloblasts.[3]

We have now analyzed KG1 and KG1a, maintained in one of our laboratories (Leukaemia Research Fund Centre) for a period of 2 years, with a battery of monoclonal antibodies and with molecular probes for rearrangement and/or expression of immunoglobulin genes, T cell receptor (TCR) α and β genes,[4,5] and the T3δ gene.[6] We report here that KG1a has undergone additional karyotypic evolution and surprisingly has a composite phenotype suggesting a possible affiliation with the T cell lineage

MATERIALS AND METHODS

Cells KG1 and KG1a cell lines[1,2] were kindly provided by Dr D. Golde, UCLA, California. They were maintained in 20% fetal calf serum in RPMI 1640 medium in a 5% CO_2-gassed incubator at concentrations between 2×10^5 and 10^6 cell/mL.

Functional studies Colony formation and responsiveness to CSF were assessed by plating between 10^3 and 10^6 cells/mL in Iscove's modified Dulbecco's medium containing 0.9% (wt/vol) methylcellulose, 5×10^{-5} mol/L 2-mercaptoethanol, 20% (vol/vol) fetal calf serum, 1% (wt/vol) delipidated and deionized bovine serum albumin, and 0.3 mg human transferrin/mL saturated with $FeCl_3$. Conditioned media from the 5637 bladder carcinoma line, from phytohemagglutinin (PHA)-stimulated lymphocytes, and from human placenta were used as sources of CSF.[7] Inducers of differentiation used were 12-0-tetradecanoylphorbol-13-acetate (TPA), purified thymosin fraction V (a kind gift from Dr A.L. Goldstein, Department of Biochemistry, The George Washington University Medical Center, Washington, DC) and purified interleukin 1 (IL 1) protein (Lab Impex UK, London).

Karyotype. The chromosomal characteristics of KG1 and KG1a were determined by standard Giemsa banding using a modification of the method of Gallimore and Richardson.[8]

Immunophenotype The cell surface immunophenotype of KG1 and KG1a was characterized with a series of murine monoclonal antibodies (see Table 2). These were either directly labeled with fluorochromes (Leu-series from Becton Dickinson) or were unlabeled and detected by fluorescein-labeled goat antimouse Ig Binding of antibodies was assessed by flow cytometry (Becton Dickinson, FACS 440)

Nuclear terminal deoxynucleotidyl transferase (TdT) was assayed by indirect immunofluorescence using an affinity-purified rabbit anti-TdT kindly provided Dr F Bollum (Uniformed Services University of the Health Sciences, Md) Cytoplasmic staining with monoclonal antibodies was carried out on methanol-fixed cytospin preparation as for TdT.[9]

Isolation and analysis of RNA Total cellular RNA was isolated from cells using the guanidinium isothiocyanate procedure of Chirgwin et al.[10] RNA was prepared for electrophoresis in 1 1% agarose gels by denaturation in glyoxal and dimethyl sulfoxide (DMSO) at 50 °C.[11] Transfer to nitrocellulose, hybridization, washing, and autoradiography after electrophoresis were essentially as described for DNA analysis (see the following material).

Description of probes Analysis of TCR β chain gene was carried out using a constant region (*Bgl*II-*Stu*I fragment) cDNA probe isolated from the leukemic cell line Jurkat.[5] This probe and a TCR α chain cDNA probe[4] were kind gifts from Dr T.W Mak (Ontario Cancer Institute).

Immunoglobulin gene analysis was also carried out using two probes a J_H region probe (*Bam*HI-*Hind*III), which was a gift from Dr P. Leder,[12] and a constant region (Cμ) cDNA probe isolated from the B cell line RPMI 1788 (Molgaard and Ford, unpublished work).

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Expression of the T3 δ chain gene was detected using a cDNA probe that has been previously described.[5] A murine genomic probe for actin[13] that cross-hybridizes efficiently with human DNA/RNA was used as a positive control in all Northern blotting experiments.

Isolation and analysis of DNA. Total genomic DNA was extracted from the cells by lysis in 0.5% sodium dodecyl sulfate (SDS) and treatment with proteinase K and RNAse, followed by phenol extraction and ethanol precipitation essentially as described by Ford et al.[14]

Purified DNA was digested with restriction endonucleases according to the recommendation of the suppliers and then fractionated by electrophoresis through 0.5% to 0.9% agarose gels and transferred to nitrocellulose. DNA probes were nick-translated and hybridized to nitrocellulose-bound DNA. After hybridization, blots were washed twice for 30 minutes each in 0.1× SSC (15 mmol/L sodium chloride, 1.5 mmol/L sodium citrate) and 0.1% SDS (sodium dodecyl sulfate) at 68 °C. Hybridization was visualized by conventional ^{32}P autoradiography as described by Ford et al.[14]

RESULTS

Karyotypic evolution of KG1a. The banded karyotype characteristics of KG1 and KG1a are summarized in Table 1. These data indicate that our KG1 cell line is a derivative of that originally described by Koeffler and Golde[1] but has acquired additional karyotypic abnormalities. KG1a initially had an identical karyotype to the parental line KG1.[2] However, it is clear that the KG1a line carried in our laboratory, though certainly a clonal derivative of KG1, has acquired several additional chromosome alterations including the new markers t(19;?) (q13;?) and i(11q) (qter→cen→qter).

Cell surface immunophenotyping of KG1 and KG1a. Table 2 summarizes the immunophenotypic investigations on KG1 and KG1a using a series of monoclonal antibodies detecting cell surface antigens associated with, though not necessarily exclusive to, particular hematopoietic lineages.

KG1 has a composite phenotype consistent with these cells being arrested at the myeloblastic stage of myeloid differentiation[15] as initially reported by Koeffler et al.[1,2] The cell surface immunophenotype of KG1a differs from that of KG1 with respect to four antigenic sites (Table 2, Fig 1). In addition to lacking HLA-DR/DP as originally reported,[2] KG1a does not express the pan-myeloid antigen MY9[15] but does express the early T cell–associated antigen CD7(gp40).[18] KG1a cells consistently react better with the CD7 monoclonal antibody Leu-9[19] than with the CD7 antibody WT1.[18] These antibodies probably detect different epitopes on the same gp40 structure.[20] The latter antigen is sometimes expressed in acute myelogenous leukemia (AML)[18,20] but is not detectably present in KG1. Other T and B lineage–restricted antigens (and TdT) are not present on the cell surface of KG1 or KG1a cells (Table 2). In contrast to KG1, KG1a does not express the antigen detected by OKT10.[21] This antibody detects a glycoprotein of 45kd encoded on chromosome 4[22] that is present on all myeloid colony–forming cells,[23] thymocytes, acute lymphocytic leukemia (ALL), and AML.[24] KG1a has a very weak expression of the X hapten (3-fucosyl-*n*-acetyl-lactosamine) epitopes detected by Leu-M1 and My1[25,26] but, in common with KG1, expresses the myeloid antigen MY7.[15] The latter is described

Table 1. Karyotypic Features of KG1 and KG1a Cell Lines

Chromosome	KG1a (No. Copies) Normal	KG1a (No. Copies) Abnormal	KG1 (No. Copies) Normal	KG1 (No. Copies) Abnormal
1	2	+1 mar 1^1	2	+1 mar 1^2
2	2		2	+1 mar 2
3	2		2	
4	1	+1 mar 3 (+1 mar 7)	1	+1 mar 3 (+1 mar 7)
5	1		1	
6	2		2	
7	1	+1 mar 4*	1	+1 mar 4
8	1	+1 mar 5	1	+1 mar 5
9	2		2	
10	2		2	
11	2	+1 mar 6	2	
12	1		1	
13	2		2	
14	2		2	
15	2		2	
16	2		2	
17	1		1	+1 mar 7
18	2	+1 mar 7	2	
19	2	+1 mar 8	2	
20	2		2	
21	2		2	
22	1 or 2		2	
X	1		1	
Y	0 or 1		0 or 1	

Abbreviations. mar 1^1, (1p34→q11 :? :1q21→qter); mar 1^2, (1p34→q11: ? :1q21→q32); mar 2, del(2)(pter→q33); mar 3, t(4;?)(q33;?); mar 7, t(17,4)(17qter→p13.:4q21→qter); mar 4, del(7)(pter→q22:), mar 5, t(8;?)(p22,?); mar 6, i(11)(qter→cen→qter); mar 8, t(19;?)(q13,?).

*Ten percent of the metaphases had two copies of a normal chromosome.

Table 2. Cell Surface Immunophenotypes of KG1 and KG1a

Antibodies*	KG1	KG1a
Myeloid		
Leu M1, My1, (CD$_w$15)	+	+ (w)
MY7, MCS-2, (CD$_w$13)	+	+
MY9	+	−
LICR LON R10 (Antiglycophorin)	−	−
J15 plus AN51 (antiplatelet glycoproteins)/(IIb/IIa and I)	−	−
T lineage		
Leu-1 (CD5)	−	−
Leu-2[a] (CD8)	−	−
Leu-3 (CD4)	−	−
Leu-4/UCHT1 (CD3)	−	−
Leu-5/OKTII (CD2)	−	−
Leu-6/NA134 (CD1)	−	−
Leu-8	−	−
Leu-9/WT1 (CD7)	−	+
OKT17 (pan-T)	−	−
B lineage		
Leu-12/B4/HD-37 (CD19)	−	−
BB1	−	−
BB2	−	−
Pan-lymphoid		
Campath 1	−	−
TdT†	−	−
IL 2 receptor, anti-Tac	−	−
Pan hematopoietic precursor		
OKT10	+	−
RFB-1	+	+
3C5, My10	+	+
NK cell markers		
Leu-7	−	−
Leu-11A	−	−
Others		
HLA-DR/DP: DA2,CA1	+	−
HLA-AB: W6/32	+	+
2D1/T200 (leu common)	+	+
Thy 1	−	−
Transferrin receptor: OKT9 antitransferrin receptor (BD)	+	+
Anti-CALLA (BD); AL-2	−	−

Key: +, >50% cells staining; −, <5% cells staining; W, very weakly staining; CD, cluster of differentiation nomenclature[16]; IL 2, interleukin 2; NK, natural killer; CALLA, common acute lymphoblastic leukemia.

*See refs 15 to 17 for review and details.

†Nuclear staining in positive controls (acute lymphoblastic leukemia cell lines).

w—very weak staining.

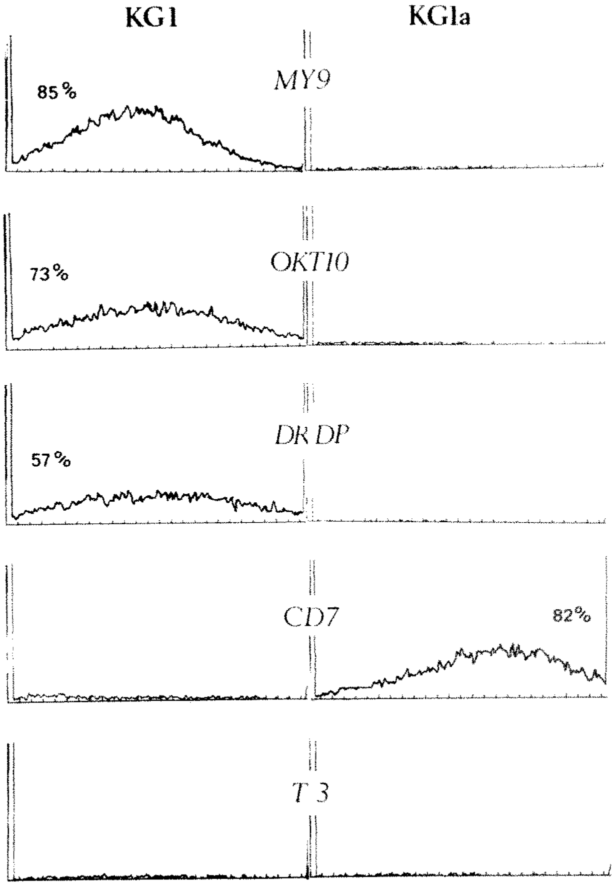

Fig 1. Flow cytometry of cell surface antigens on KG1 and KG1a cells. Viable cells were stained in suspension with monoclonal antibodies that were either directly labeled with fluorochrome or unlabeled and followed by fluorochrome-labeled anti-mouse Ig. Vertical axis, relative cell number; horizontal axis, relative fluorescence intensity. The percent positive cells above the background value (with Ig class-specific controls) are given for each antibody.

as a myelomonocytic cell-associated protein, the expression of which is probably regulated in concert with cell proliferation.[15] MY7 is considerably more restricted to the myeloid lineage than is My1/Leu-M1 and is the only marker on KG1a cells that might indicate that these could be myeloid.

Molecular phenotypes of KG1 and KG1a. Table 3 and Fig 2 summarize the data obtained from Southern and Northern blot analyses of DNA and RNA derived from KG1 and KG1a with probes specific for immunoglobulin or TCR complex genes. An actin probe was used as a positive RNA control for all cell lines and the myeloblastic cell line ML1 and the T-ALL cell line Molt-13 (kindly provided by Dr J. Minowada) included for comparison. Both KG1 and KG1a have germline configurations of IgH and TCRβ genes (data not shown). However, both KG1 and KG1a have low-level transcription of IgH.

Paradoxically the myeloid cell line ML1 used as a control has a rearranged μ allele,[27] but no transcripts were detectable. KG1a cells have TCRβ gene mRNA, though present at low levels (arrows in Fig 2) compared with most T leukemia cell lines (cross-reference Molt 13, a T-ALL cell line, Fig 2). A trace of TCRβ mRNA was also detectable in KG1. No T cell receptor α-chain gene mRNA was detectable in KG1 or in KG1a when compared with a T leukemia cell line control (data not shown).

KG1a had abundant T3δ mRNA of the same size (0.8 to 0.9 kb) as in normal T cells and T leukemic cells.[5] KG1 cells had no detectable T3δ mRNA (Fig 2). Since assembly of a functional TCR-T3 complex requires TCRα, β, and T3 gene products to be coordinately expressed,[28-30] the lack of cell suface T3 (CD3) probably reflects the absence of TCRα transcripts and protein. It was therefore likely that T3δ chain

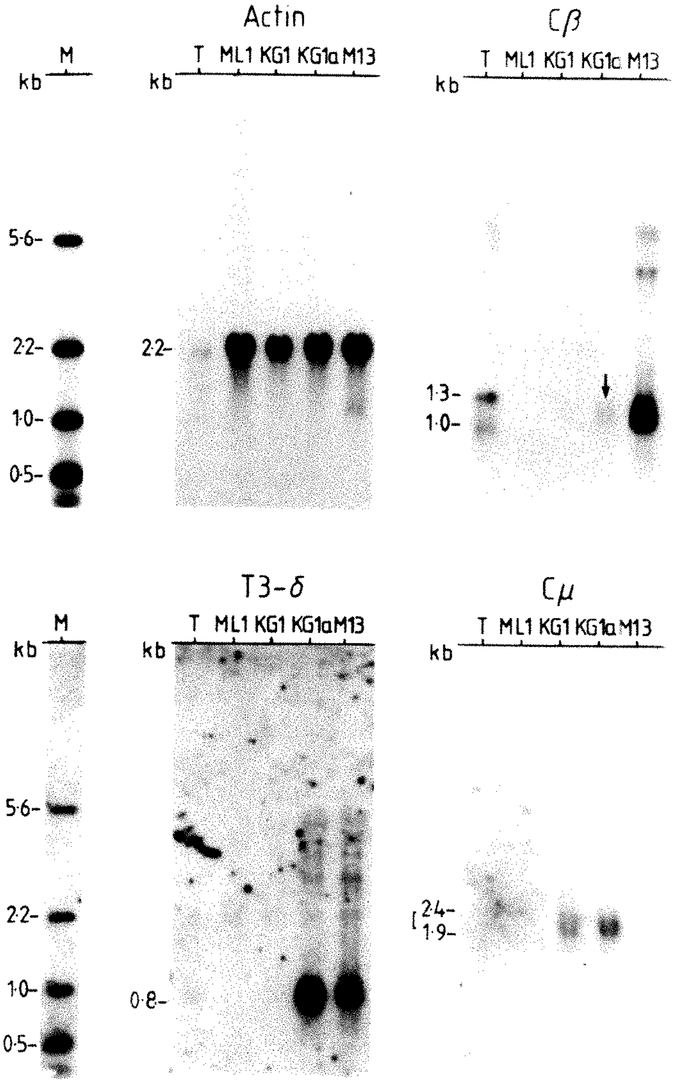

Fig 2. Expression of TCR-T3 and Ig genes in KG1 and KG1a cells by RNA-transfer analysis of RNAs extracted from hematopoietic cell lines and fresh leukemic cells. Ten micrograms of total cellular RNA from each sample were electrophoresed and transferred to a nitrocellulose filter (see Materials and Methods). The nitrocellulose-bound RNA was then hybridized to a series of radiolabeled DNA probes: actin, a control for the relative amount of RNA present; Cβ, the constant region of the TCRβ gene; T3δ, the δ chain gene of the T3 complex; and Cμ, the constant region of the Igμ gene. After each hybridization and autoradiographic visualization the probe was removed from the filter with 100 °C H_2O. Abbreviations: M, molecular weight markers with sizes given in kilobases (kb); T, a mature T cell leukemia; ML1, a myeloid cell line with a rearranged IgH allele[27]; KG1, KG1a, see the text; M13, Molt 13, a pre-T acute leukemic line (J. Minowada, personal communication). Note that the mature T cell leukemia had a low proliferative status and was only weakly positive for cell surface CD3; mRNA signals for actin and TCRβ and T3δ are correspondingly weak but are included to provide relevant molecular weight markers of TCR and T3δ transcripts (arrows). The weak TCRβ signal with KG1a DNA cannot be ascribed to spill-over from the adjacent track containing T cell line (M13) DNA because the same result was obtained in four other independent experiments in which no T cell control was run in juxtaposition to the KG1a DNA digest. The identity of the high-molecular weight bands seen with the TCRβ probe in M13 T cell line DNA and with T3δ (KG1a and M13 DNA) is unknown. One possibility is that they represent unspliced messenger RNA species.

protein was being synthesized but not incorporated into the cell surface. This was confirmed by intracellular staining of KG1a cells with monoclonal antibodies to T3δ (Fig 3). More than 95% of KG1a cells contained cytoplasmic T3δ. Staining intensity varied considerably between individual cells, and in the brighter cells, it appeared that the T3δ protein had a perinuclear localization (arrows in Fig 3A) that we presume reflects accumulation in the perinuclear envelope–endoplasmic reticulum. KG1 cells were not specifically stained with anti-T3δ antibodies (Fig 3D). Occasional dead cells stained nonspecifically, and the level of background staining, though higher than that seen with KG1a, was no different from that in controls with normal IgG or anticytokeratin. KG1 cells therefore do not appear to synthesize T3 proteins in accord with the lack of detectable T3δ mRNA.

Table 3. Molecular Phenotypes of KG1 and KG1a

	KG1		KG1a	
Probes	DNA	mRNA	DNA	mRNA
1. Immunoglobulin	G-L	+ weak	G-L	+ weak
J_H		(1.9–2.4 kb)		(1.9–2.4 kb)
2. TCR complex				
β gene	G-L	–*	G-L	+ weak
				1.0–1.3 kb
α gene	NA	–	NA	–
T3δ gene	NA	–	NA	+(0.8–0.9 kb)

Abbreviations: G-L, germline; NA, not assessed; –, not detected.

*A trace band at around 1 to 1.3 kb was just visible in some experiments.

Functional activity of KG1 and KG1a. Using relatively early-passage KG1 cells we were able to confirm the original reported difference between KG1 and KG1a with respect to CSF-dependent colony formation. No colonies were formed by KG1a with or without added conditioned medium, whereas KG1 did form colonies with conditioned medium, albeit at a lower level than that originally reported: 40 to $90/10^4$ cells plated compared with 300 to $350/10^4$.[2] KG1 cells differentiated into adherent macrophages in the presence of 1.7×10^{-7} mol/L TPA whereas KG1a was unresponsive, as reported previously.[2]

In view of the expression by KG1a cells of some characteristics associated with immature T cells, we attempted to

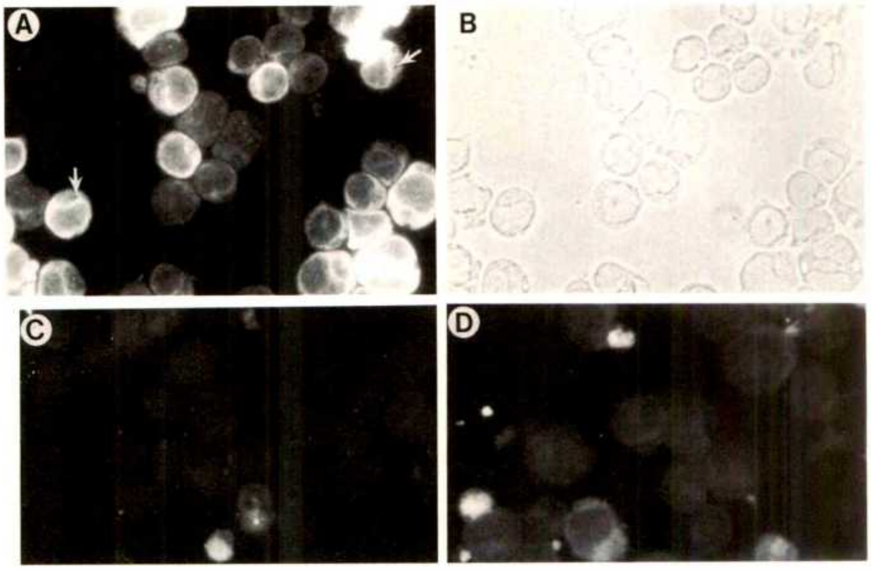

Fig 3. Intracytoplasmic T3δ chains in KG1a but not in KG1 cells. (A) KG1a stained with anti-T3δ monoclonal antibodies (see the following material). (B) KG1a, phase contrast (same field as A). (C) KG1a, control staining with anticytokeratin antibody (Le 61). (D) KG1 stained with anti-T3δ monoclonal antibodies. Cells were smeared using cytospin apparatus (Shandon, Cheshire, UK) and fixed in methanol. Slides were then incubated at room temperature with the following sequence: either a cocktail of three different anti-T3δ monoclonal antibodies, SP-64, SP-78, SP-34[6,31] or a monoclonal antibody to cytokeratin Le 61 (as control) followed by goat antimouse Ig and sheep antigoat Ig. The latter two antibodies were labeled with fluorescein isothiocyanate.

induce the expression of additional T cell lineage markers. KG1a cells were incubated with purified thymosin fraction V peptide (up to 100 μg/mL) or purified interleukin 1 (up to ten units) either singly or in combination. Over a period of four days these additives failed to induce the expression of cell surface T1(CD5), T3(CD3), or T11(CD2).

DISCUSSION

The KG1 cell line, established from a patient with erythroleukemia in myeloblastic relapse, has the cytochemical, functional, and immunologic characteristics of myeloblasts.[1-3]

KG1a, a subline of KG1, was originally reported to have lost these features and to have an undifferentiated or immature myeloid phenotype.[2,3] Our data confirm these earlier observations but somewhat surprisingly show that KG1a cells express three markers normally associated with immature T cells and their leukemic counterparts in T-ALL. These are expression of the TCRβ gene and T3δ gene mRNAs, the presence of cytoplasmic but not cell surface CD3 protein, and the presence of the cell surface antigen gp40/CD7.[18,29,30] KG1a cells have no cell surface T3 antigen detectable by immunofluorescent staining and flow cytometry but accumulate T3 proteins intracytoplasmically with a prominent perinuclear localization. Elsewhere[30] we describe that this is a consistent characteristic of leukemic and normal T cell precursors and reflects restriction to the site of synthesis of T3 proteins in the perinuclear endoplasmic reticulum and defective transport to the golgi and cell surface in the absence of TCRα chains (or α/β dimers).

Only low levels of TCRβ mRNA have been detected in KG1a cells. These cells also have the germline configuration of the TCRβ gene that is also normally seen in the immature T3/T6⁻ (CD3/CD1⁻) thymocytes.[29,30] It is not clear, however, whether TCRβ genes are transcribed prior to rearrangement in normal T cell development, and the only example, other than KG1a, of apparent germline gene transcripts is in cells from an individual patient with T-ALL.[30] It is possible, however, that a minor subpopulation of KG1a cells have rearranged the TCRβ gene and that these cells are transcribing the TCRβ mRNA detected. Such a rearrangement would necessarily be aberrant since the normal size (1.0 kb or 1.3 kb) transcripts[5] are not detected in these cells.

A trace of TCRβ mRNA was also detected in some but not all experiments with KG1. This lack of reproducibility prohibits any clear interpretation, but one possibility is that a minor fraction of the parental KG1 cells shares with the KG1a subline the characteristic of transcribing TCRβ.

KG1a appears so far to be unique as a myeloid line derivative in expressing both TCRβ and T3δ genes. Although AML cells may occasionally show partial rearrangement of TCRβ,[32] we have observed no TCRβ rearrangement or expression (mRNA) or T3δ expression (mRNA and/or cytoplasmic protein) in other myeloid leukemia cell lines (EM2, EM3, HL-60, U937, THP-1) or in 26 uncultured AML (A.J.F. et al, unpublished observations).

Although KG1a cells have a composite phenotype similar to T cell precursors and equivalent leukemic cells, they are TdT-negative and could not be induced to express additional T cell characteristics. KG1 and KG1a both express Igμ chain mRNA transcribed from a germline IgH gene. Transcription of unrearranged Ig genes has previously been described in murine B, T, and myeloid cells.[33-35] Some human myeloid leukemias may have partially rearranged IgH genes,[27,32,36,37] but little data are available on μ gene transcription. The AML cell lines ML1, 2, and 3 have a common μ gene rearrangement[27,37] (A.J.F., unpublished observation) but fail to produce any detectable μ mRNA (Fig 2). Similarly, one AML patient's cells investigated by Cheng et al[32] had μ gene rearrangement but no transcripts. Since neither KG1 nor KG1a express antigenic markers of B cell precursors (eg, CD19), there are no other indications that they might have B lineage affiliation.

There are two interpretations of these observations. First, KG1a being an aneuploid leukemia cell line maintained in vitro for more than 4 years now expresses an aberrant phenotype that has no distinctive lineage affiliation.[38] This would perhaps accord with the observation that KG1a still expresses one myeloid-associated antigen (MY7) and transcribes μ genes in the germline configuration as well as TCRβ.

Second, this patient's leukemia, and the original KG1 cell line, were in fact derived from a common myeloid–T lymphoid cell precursor, and a subline (KG1a) had evolved from a daughter cell committed to the T cell lineage. The existence of such a bipotential or multipotential progenitor cell has been deduced from studies on murine hematopoiesis[39,40] and on chronic granulocytic leukemia in humans. Fialkow and colleagues have provided evidence for clonal involvement of mature T cell in some cases of CML,[41] and we have recently described a case of Ph^1-positive CML that evolved into a T cell blast crisis expressing the composite phenotype of thymocytes including a clonally rearranged TCRβ gene and a rearranged bcr (Ph^1 breakpoint region.)[42]

At present it is not possible to decide which of these interpretations is correct. Other strategies are being adopted to induce differentiation in KG1a cells, which might resolve the issue. KG1a has chromosomal features confirming its clonal origin from KG1, but in contrast to the earlier report,[2] we find that it now has a distinctive karyotype. It may be significant that one of the two new karyotypic features of KG1a is an isochromosome 11q. Since the T3δ gene maps to 11(q23-qtre),[43] it is certainly possible that the two additional copies of 11q are responsible for the T3δ gene transcripts detected and that this karyotypic change is in part responsible for the dramatic shift in phenotype between KG1 and KG1a.

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REFERENCES

1. Koeffler HP, Golde DW: Acute myelogenous leukemia: A human cell line responsive to colony-stimulating activity. Science 200:1153, 1978

2. Koeffler HP, Billing R, Lusis J, Sparkles R, Golde DW: An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1). Blood 56:265, 1980

3. Koeffler HP: Study of differentiation and proliferation of leukemic cells using myeloid leukemia cell lines, in Bloomfield (ed): Chronic and Acute Leukemias in Adults. Boston, Martinus Nijhoff, 1985, p 27

4. Yanagi Y, Chan A, Chin B, Minden M, Mak TW: Analysis of cDNA clones specific for human T cells and the β and α chains of the T cell receptor heterodimer from a human T-cell line. Proc Natl Acad Sci USA 82:3430, 1985

5. Yoshikai Y, Anatoniou D, Clark SP, Yanagi Y, Sangster R, van den Elsen R, Terhorst C, Mak TW: Sequence and expression of transcripts of the human T-cell receptor β chain genes. Nature 312:521, 1984

6. van den Elsen P, Shepley B, Borst J, Coligan JE, Markham AF, Orkin S, Terhorst C: Isolation of the DNA clones encoding the 20kd T3 glycoprotein of the human T-cell receptor complex. Nature 312:413, 1984

7. Myers CD, Katz FE, Joshi G, Millar JL: A cell line secreting stimulating factors for CFU-GEMM culture. Blood 64:152, 1984

8. Gallimore PH, Richardson PR: An improved banding technique exemplified in the karyotype analysis of two strains of rat. Chromosoma 41:259, 1973

9. Bollum F: Terminal deoxynucleotidyl transferase as a hemopoietic cell marker. Blood 54:1203, 1979

10. Chirgwin JM, Pryzbyla AE, Macdonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 18:5294, 1979

11. Thomas P: Hybridization of denatured RNA transferred or dotted onto nitrocellulose paper. Methods Enzymol 100:255, 1983

12. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P: Structure of the human immunoglobulin locus: Characterization of embryonic and rearranged J and D genes. Cell 27:583, 1981

13. Willison KR, Dudley K, Potter J: Molecular cloning and sequence analysis of a haploid expressed gene encoding t complex polypeptide 1. Cell 44:727, 1986

14. Ford AM, Molgaard HV, Greaves MF, Gould HJ: Immunoglobulin gene organisation and expression in haemopoietic stem cell leukaemia. EMBO J 2:997, 1983.

15. Griffin JD: Surface marker analysis of acute myeloblastic leukemia, in Bloomfield CD (ed): Chronic and Acute Leukemias in Adults. Boston, Martinus Nijhoff, 1985, p 133

16. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF (eds): Leucocyte Typing. Berlin, Springer Verlag, 1984

17. Greaves MF, Delia D, Newman RA, Vodinelich L: Analysis of leukaemic cells with monoclonal antibodies, in McMichael AJ, Fabre JW (eds): Monoclonal Antibodies in Clinical Medicine. Orlando, Fla, Academic, 1982, p 129

18. Vodinelich L, Tax W, Bai Y, Pegram S, Capel P, Greaves MF: A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 62:1108, 1983

19. Link M, Warnke R, Finlay J, Amylon M, Miller R, Dilley J, Levy R: A single monoclonal antibody identifies T-cell lineage of childhood lymphoid malignancies. Blood 62:722, 1983

20. Sutherland DR, Rudd CE, Greaves MF: Isolation and characterization of a human T lymphocyte associated glycoprotein (gp40). J Immunol 133:327, 1984

21. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF: Discrete stages of human intrathymic differentiation: Analysis of normal thymocytes and leukemic lymphoblasts of T lineage. Proc Natl Acad Sci USA 77:1588, 1980

22. Katz F, Povey S, Parker M, Schneider C, Sutherland R, Stanley K, Solomon E, Greaves MF: Chromosome assignment of monoclonal antibody defined determinants on human leukaemic cells. Eur J Immunol 13:1008, 1983

23. Sieff C, Bicknell D, Caine G, Robinson J, Lam G, Greaves MF: Changes in cell surface antigen expression during hemopoietic differentiation. Blood 60:703, 1982

24. Greaves MF, Delia D, Sutherland R, Rao J, Verbi W, Kemshead J, Robinson J, Hariri G, Goldstein G, Kung P: Expression of the OKT monoclonal antibody defined antigenic determinants in malignancy. Int J Immunopharmacol 3:283, 1981

25. Huang LC, Civin CI, Magnani JL, Shaper JH, Ginsburg V: My-1, the human myeloid-specific antigen detected by mouse monoclonal antibodies, is a sugar sequence found in lacto-*N*-fucopentaose III. Blood 61:1020, 1983

26. Gooi HC, Thorpe SJ, Hounsell EF, Rumpold H, Kraft D, Forster O, Feizi T: Marker of peripheral blood granulocytes and

monocytes of man recognised by two monoclonal antibodies VEP8 and VEP9 involves the trisaccharide 3-fucosyl-*N*-acetyllactosamine. Eur J Immunol 13:306, 1983

27. Palumbo A, Minowada J, Erikson J, Croce CM, Rovera G: Lineage infidelity of a human myelogenous leukemia cell line. Blood 64:1059, 1984

28. Ohashi P, Mak TW, van den Elsen P, Yanagi Y, Yoshikai Y, Calman A, Terhorst C, Stobo J, Weiss A: Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. Nature 316:606, 1985

29. Royer HD, Ramarli D, Acuto O, Campen TJ, Reinherz EL: Genes encoding the T-cell receptor β and α subunits are transcribed in an ordered manner during intrathymic ontogeny. Proc Natl Acad Sci USA 82:5510, 1985

30. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, van den Elsen P, Gold D, Terhorst C, Greaves MF: Developmentally regulated rearrangement and expression of genes encoding the T cell receptor–T3 complex. Cell 46:75, 1986

31. Pessano S, Oettgen H, Bhan AK, Terhorst C: The T3/T cell receptor complex: Antigenic distinction between the two 20-Kd T3 (T3δ and T3ϵ) subunits. EMBO J 4:337, 1985

32. Cheng GY, Minden MD, Toyonaga B, Mak TW, McCulloch EA: T cell receptor and immunoglobulin gene rearrangements in acute myeloblastic leukemia. J Exp Med 163:414, 1986

33. Kemp DJ, Harris AW, Adams JM: Transcripts of the immunoglobulin Cμ gene vary in structure and splicing during lymphoid development. Proc Natl Acad Sci USA 77:7400, 1980

34. Yancopoulos GD, Alt FW: Developmentally controlled and tissue-specific expression of unrearranged V_H gene segments. Cell 40:271, 1985

35. Spalding DM, Griffin JA: Different pathways of differentiation of pre-B cell lines are induced by dendritic cells and T cells from different lymphoid tissues. Cell 44:507, 1986

36. Rovigatti U, Mirro J, Kitchingman G, Dahl G, Ochs J, Murphy S, Stass S: Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia. Blood 63:1023, 1984

37. Ha K, Minden M, Hozumi N, Gelfand EW: Immunoglobulin gene rearrangement in acute myelogenous leukemia. Cancer Res 44:4658, 1984

38. Smith LJ, Curtis JE, Messner HA, Senn JS, Furthmayr H, McCulloch EA: Lineage infidelity in acute leukemia. Blood 61:1138, 1983

39. Abramson S, Miller RC, Phillips RA: The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J Exp Med 145:1567, 1977

40. Keller G, Paige C, Gilboa E, Wagner EF: Expression of a foreign gene in myeloid and lymphoid cells derived from multipotential haematopoietic precursors. Nature 318:149, 1985

41. Fialkow PJ, Denman AM, Jacobson RJ, Lowenthal MN: Chronic myelocytic leukemia: Origin of some lymphocytes from leukemic stem cells. J Clin Invest 62:815, 1978

42. Chan LC, Furley AJ, Ford AM, Yardumian DA, Greaves MF: Clonal rearrangement and expression of the T cell receptor β gene and involvement of the break point cluster region (bcr) in blast crisis of CGL. Blood 67:533, 1986

43. van den Elsen P, Bruns G, Gerhard DS, Pravtcheva D, Jones C, Housman D, Ruddle FA, Orkin S, Terhorst C: Assignment of the gene coding for the T3-δ subunit of the T3–T-cell receptor complex to the long arm human chromosome 11 and to mouse chromosome 9. Proc Natl Acad Sci USA 82:2920, 1985

A Novel Basis for δβ-Thalassemia in a Chinese Family

By George F. Atweh, Ding-er Zhu, and Bernard G. Forget

We have studied a Chinese family in which β-thalassemia and δβ-thalassemia were found in simple and compound heterozygous states. The δβ-thalassemia heterozygote (the mother) had 22.3% hemoglobin F, of which 40% was $^{G}\gamma$ and 60% $^{A}\gamma$; globin chain studies showed an $\alpha/\beta + \gamma$ ratio of 1.36. The compound heterozygote for δβ-thalassemia and β-thalassemia (the child) had the clinical picture of thalassemia intermedia and an $\alpha/\beta + \gamma$ ratio of 4.44. Gene mapping studies were performed using DNA from the affected child. Seventy kilobases of DNA in the β-globin gene cluster starting upstream from the ε-globin gene and ending downstream from the β-globin gene were mapped, and no detectable deletions or rearrangements were detected. In addition, heterozygosity was detected at multiple polymorphic restriction sites in and 3′ to the β-globin gene, which excludes the possibility of a deletion of the entire β-globin gene cluster. This is the first example of a nondeletion δβ-thalassemia associated with increased expression of both $^{G}\gamma$ and $^{A}\gamma$ genes.

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δβ-THALASSEMIA consists of a heterogeneous group of disorders that share a common phenotype characterized by decreased synthesis of the adult δ- and β-globin chains and increased synthesis of fetal γ-globin chains.[1] However, the increased synthesis of γ chains does not fully compensate for the lack of production of β chains, giving rise to a mild imbalance in α- to non-α-globin chain synthesis and a thalassemic phenotype.[2] The vast majority of the δβ-thalassemias that have been studied at the molecular level are associated with extensive deletions of DNA in the β-globin gene cluster that usually remove all or part of the δ- and β-globin genes in addition to varying amounts of adjacent sequences.[3,4] In this communication, we describe the interaction of a δβ-thalassemia determinant with a β-thalassemia gene in a Chinese family. Hematologic and globin synthetic studies identified simple heterozygotes for both conditions in addition to a compound heterozygote. Molecular studies did not show any detectable deletions or rearrangements in the β-globin cluster associated with the δβ-thalassemia phenotype. We discuss the implications of these findings for the understanding of hemoglobin switching and speculate on a possible molecular mechanism that could explain this phenotype.

MATERIALS AND METHODS

Summary of case histories. The index case was a 4-year-old Chinese boy who immigrated to the United States with his father and mother at the age of 3 years. His early childhood was notable for easy fatiguability and excessive sleep but was otherwise unremarkable. On a routine evaluation for admission to a day care center in this country, he was noted to be pale. Physical examination revealed a grade II/VI systolic ejection murmur and a spleen that extended about 5 cm below the left costal margin. Both the father and mother were healthy, and the child had no siblings. Table 1 summarizes the hematologic data in this family.

Globin studies. Hemoglobin electrophoresis with HbA_2 and HbF quantitation were performed using standard methods.[5] The distribution of HbF in red cells was assessed as described by Kleihauer et al.[6] Globin chain synthesis in peripheral blood reticulocytes was studied using carboxymethyl cellulose chromatography as previously described.[7] The $^{G}\gamma$ and $^{A}\gamma$ composition of the fetal hemoglobin was further analyzed using high-performance liquid chromatography (HPLC)[8] and polyacrylamide–urea gel electrophoresis.[9]

Molecular studies. High-molecular weight DNA was isolated from peripheral blood leukocytes using the procedure of Blin and Stafford.[10] DNA was digested using different restriction enzymes, fractionated by electrophoresis in agarose gels, blotted onto nitrocellulose filters, and hybridized to nick-translated DNA probes as described by Southern.[11] Different unique sequence probes from the ε-, γ-, ψβ1- and β-globin genes were used in the gene mapping experiments. Restriction enzyme polymorphisms in the β-globin gene cluster were evaluated using the following enzyme/probe combinations[12]: *Hinc*II/ε gene, *Hind*III/γ gene, *Hinc*II/ψβ1 gene, *Taq*I/5′ δ-flanking DNA, *Rsa*I/5′ β-flanking DNA, *Ava*II and *Bam*H1/β gene and *Hind*III/3′ β-flanking DNA (pRK 29).[13,14]

RESULTS

Globin studies. The results of hemoglobin and globin chain analyses are shown in Table 2. The father's hemoglobin consisted of 91.1% A, 6.5% A_2, and 2.4% F. Globin chain synthesis studies showed an α/β ratio of 1.77. These findings along with a mild microcytic anemia are typical of the β-thalassemia trait. The mother did not have anemia or microcytosis, but her blood film showed thalassemic changes. Her hemoglobin consisted of 72.6% A, 1.5% A_2, and 22.3% F. Globin chain synthetic studies (Fig 1A) showed an α/β ratio of 1.68 and an $\alpha/\beta + \gamma$ ratio of 1.36. The fetal hemoglobin distribution was heterocellular by Betke staining and the γ chain composition was 40% $^{G}\gamma$ and 60% $^{A}\gamma$. These findings are typical of δβ-thalassemia trait. The son, on the other hand, who clinically had β-thalassemia intermedia, had the following hemoglobin constitution: 15% A, 3.6% A_2, and 81.4% F. Fetal hemoglobin was heterocellularly distributed and the γ chains were 52.2% of the $^{G}\gamma$ variety and 47.8% $^{A}\gamma$. Globin chain synthesis studies (Fig 1B) showed an α/β ratio of 10.20 and an $\alpha/\beta + \gamma$ ratio of 4.44. These findings are compatible with the doubly heterozygous state for β-thalassemia and δβ-thalassemia.

Molecular studies. All of the restriction mapping experiments were performed by comparing genomic DNA from

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Table 1. Hematologic Data

	Proband	Father	Mother
Hb (g/dL)	6.8	12.2	13.2
Hct (%)	21.6	38.0	42.5
MCV (FL)	70.7	67.5	81.0
MCH (pg)	22.3	21.6	25.5
MCHC (g/dL)	31.5	32.0	31.2
Reticulocytes (%)	23.6	0.6	2.2
NRBC/100 WBC	120	0	0

Abbreviations: Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; NRBC, nucleated red blood cells.

Table 2. Results of Hemoglobin and Globin Chain Analyses

	Proband	Father	Mother
HbA (%)	15.0	91.1	76.2
HbA_2 (%)	3.6	6.5	1.5
HbF (%)	81.4	2.4	22.3
$^G\gamma/^A\gamma$* (%)	52/48	27/73	40/60
α/β†	10.20	1.77	1.68
$\alpha/\beta + \gamma$†	4.44	1.58	1.36

*Results obtained by HPLC.

†Ratio of radioactivity incorporated into globin chains that were fractionated by carboxymethyl cellulose column chromatography.

the affected child with normal DNA side by side in Southern blotting experiments. We evaluated the sizes of several overlapping restriction fragments that span more than 70 kilobases (kb) of DNA that start upstream from the ε gene and end downstream from the β gene. A summary of these results is shown in Table 3. The ε-globin region was evaluated using a genomic ε probe that hybridized to a 9.5 kb *Bcl*I fragment in both the patient's DNA and the control DNA. (Fig 2A) The γ-globin region was evaluated using a 5′ γ probe that hybridized to a 13-kb *Bgl*II fragment (Fig 2B) and three *Bam*HI fragments (15.5 kb, 5.1 kb, and 2.6 kb) in both the patient and the normal control. The ψβI-globin region was evaluated using a ψβI genomic probe that hybridized to a 15.5-kb *Bam*HI fragment and a 17.5-kb *Hind*III fragment in both the patient and normal control (Fig 2C). The δ- and β-globin regions were evaluated using a β-IVS2 probe that hybridized to an 11-kb *Xba*I fragment, (Fig 2D), a 7.6-kb *Hind*III fragment, and an 8.3-kb *Bam*HI fragment

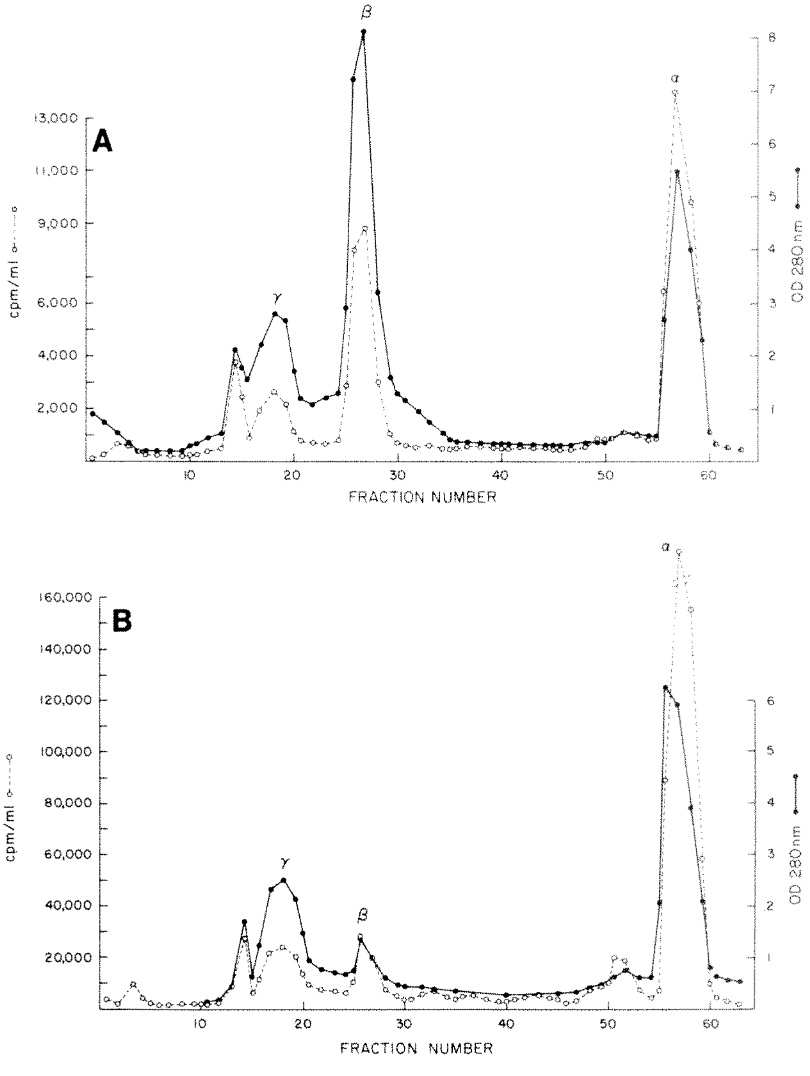


Fig 1. Fractionation on carboxymethyl cellulose of the α-, β-, and γ-globin chains prepared from whole lysates of reticulocytes incubated in the presence of ^{3}H-leucine. Solid line denotes protein concentration determined by light absorption at 280 nm; dashed line denotes radioactivity. (A) Chromatogram of globin chains from δβ-thalassemia heterozygote (mother): α/β ratio, 1.68; and α/β + γ ratio, 1.36. (B) Chromatogram of globin chains from affected child, doubly heterozygous for δβ-thalassemia and β-thalassemia: α/β ratio, 10.20; and α/β + γ ratio, 4.44

Table 3. Gene Mapping Results: Sizes of Restriction Enzyme Fragments (kb) Detected by Different Genomic Probes

	Probe				
Enzyme Digest	ϵ	γ	$\psi\beta$	β	$3'\beta$-Flanking
*Hinc*II	3.7	ND	7.6	ND	ND
*Hind*III	ND	3.4, 7.7	17.5	7.6	13.5, 15.5
*Bgl*II	10.5	13	ND	ND	ND
*Bam*HI	ND	2.6, 5.1, 15.5	15.5	8.3	ND
*Xba*I	ND	7.5, 5.1, 3.7	2, 7.5	11	ND
*Bcl*I	9.5	17.2	ND	ND	ND
*Ava*II	ND	ND	ND	2, 2.2	ND

Abbreviation: ND, not determined.

All fragment sizes were identical in patient's DNA and that of a normal control.

in both the patient and the control. These fragments in addition to others summarized in Table 3 are schematically represented on the β-globin gene cluster map in Fig 3. No abnormally sized fragments were detected in the child's DNA with any of the probes.

These results could be interpreted to indicate either the absence of any detectable deletion or rearrangement in the β-globin gene cluster or the deletion of the entire cluster on one chromosome. To distinguish these two possibilities, we looked for heterozygosity at restriction enzyme sites known to be polymorphic in the β-globin gene cluster. Of the ten restriction sites studied, the *Ava*II site in IVS-2 of the β-globin gene as well as the *Hind*III and *Bam*HI sites downstream from the β-globin gene were polymorphic (ie, both + and −) in the DNA of the affected child (see Fig 3). These findings along with the normal restriction mapping studies indicate that both β-globin gene clusters on both chromosomes are preserved with no detectable deletion or rearrangement within 70 kb of DNA. We cannot rule out a deletion or rearrangement 5′ or 3′ to this 70 kb of DNA, since unique sequence probes from these regions are not available.

To establish whether the β-globin gene on the $\delta\beta$-thalassemia chromosome is expressed, it would be necessary to establish the nature of the β-thalassemia gene on the *trans* chromosome. Analysis of restriction fragment length polymorphisms (RFLPs) in the family (Fig 3) indicates that the β-thalassemia gene is on a chromosome of the Chinese haplotype 2.[15] A number of different thalassemia mutations have been found to be associated with this haplotype in Chinese individuals, but none of these mutations could be identified by use of synthetic oligonucleotides in DNA samples from this family (H.H. Kazazian Jr, personal communication). Therefore, the β-thalassemia in this family may be due to a previously undescribed mutation, and we cannot yet ascertain whether it is of the β^0 or β^+ type.

Another noteworthy feature of the RFLP haplotype analysis in this family is the finding of an example of discordance between two sites (9 and 10 in Fig 3) that are usually in linkage disequilibrium. The nonthalassemic chromosome of the mother is associated with the absence of both the *Hind*III site (site 9) and the *Bam*HI site (site 10), whereas in an extensive study it was previously found that an absent *Bam*HI site is 100% correlated with the presence of the *Hind*III site.[16] This result could be explained either by a crossover event between the two sites (which is unlikely in view of their proximity) or by a point mutation or very small deletion event involving the *Hind*III site.

DISCUSSION

The $\delta\beta$-thalassemia phenotype of the Chinese family reported in this communication is characterized by an elevated level of fetal hemoglobin (22.3%) in the heterozygous state. The γ-globin chains consist of 40% ${}^{G}\gamma$ and 60% ${}^{A}\gamma$. The increase in γ-globin gene expression, however, is not enough to compensate for the decrease in β-globin gene expression as demonstrated by the imbalance between α-and non–α-globin chain synthesis. This is reflected by the thalassemic morphology of the RBC in the heterozygous state and a marked anemia when $\delta\beta$-thalassemia coexists with β-thalassemia in a compound heterozygous state. It is unclear whether the interacting β-thalassemia gene in this case is of the β^0 or β^+ variety, since no β-thalassemia homozygotes exist in this family. Thus, in the absence of a homozygous state for this type of $\delta\beta$-thalassemia, it cannot be determined whether the

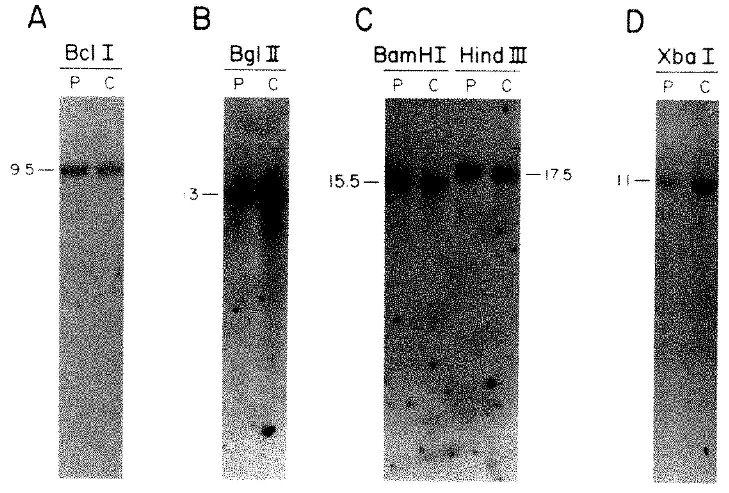
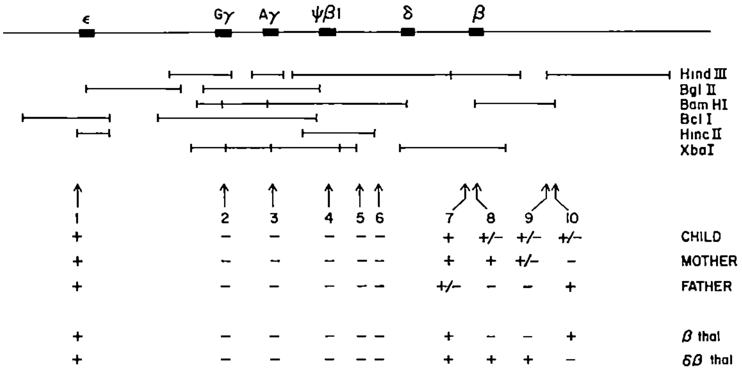


Fig 2. Mapping of restriction enzyme sites in the β-globin gene cluster of the patient doubly heterozygous for β-thalassemia and $\delta\beta$-thalassemia. The patient's DNA (P) and DNA from a normal control (C) were digested with different restriction enzymes as indicated above the autoradiographs and analyzed by Southern gel blotting. (A) A 9.5-kb *Bcl*I fragment when a 1.3-kb *Bam*HI/*Eco*RI ϵ probe is used. (B) A 13-kb *Bgl*II fragment when a 2.7-kb *Eco*RI 5′ ${}^{A}\gamma$ probe is used. (C) A 17.5-kb *Hind*III fragment and 15.5-kb *Bam*HI fragment when a 4.2 kb *Bgl*II $\psi\beta1$ probe is used. (D) An 11-kb *Xba*I fragment when a 0.95-kb *Bam*HI/*Eco*RI β-IVS2 probe is used.


ε
Gγ
Aγ
ψβ1
δ
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Hind III
Bgl II
Bam HI
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Fig 3. A map of the β-globin gene cluster. The lines under the map represent the DNA fragments detected in DNA from the patient doubly heterozygous for β-thalassemia and $\delta\beta$-thalassemia when the different restriction enzymes and probes summarized in Table 3 were used in Southern blotting experiments. All of the overlapping fragments are identical in size to those detected in the normal control. The numbered arrows represent the positions of polymorphic sites that were identified by the following restriction endonculeases: 1, 4 and 5, *Hinc*II; 2, 3 and 9, *Hind*III; 6, *Taq*I; 7, *Rsa*I; 8, *Ava*II; and 10, *Bam*HI. A + indicates cleavage and a –, absence of cleavage at a site.

β-globin gene on the $\delta\beta$-thalassemia chromosome is partially or completely silenced. This will have to await the cloning of the β-thalassemia gene from this chromosome and its study in an in vitro expression system. The expression of the δ-gene on the $\delta\beta$-thalassemia chromosome is also probably decreased or absent as suggested by a low hemoglobin A_2 level of 1.5% in the heterozygous mother.

$\delta\beta$-thalassemia and HPFH, both disorders characterized by the failure of the normal switch from fetal to adult globin production, have been the subject of extensive molecular studies over the last several years. It became apparent during the earlier stages of these studies that some cases of HPFH were associated with extensive deletions in the β-globin cluster,[17-20] whereas others were associated with a grossly normal β-globin cluster.[18,21] The former were called deletion HPFH and the latter nondeletion HPFH. On the other hand, all but one example of $\delta\beta$-thalassemia were associated with extensive DNA deletions.[3,4] Multiple theories have been suggested to explain the phenotype of HPFH and $\delta\beta$-thalassemia on the basis of these deletions. First it was suggested that the extensive deletions may remove a negative regulatory element between the $^{A}\gamma$- and δ-genes and result in the continued expression of the fetal genes into adult life.[22] Others suggested that the deletions may affect the chromatin structure in the β-globin cluster and maintain the fetal genes in a transcriptionally active domain that results in the continued expression of the fetal genes into adult life.[23] Yet another more recent theory is that the deletion may result in enhanced expression of the fetal genes by bringing into the neighborhood of these genes DNA sequences that function as enhancer elements.[24]

The nondeletion HPFH syndromes have been studied more recently by several groups,[25-27] and point mutations in the promoter regions of the γ-globin genes were identified and shown to be tightly linked to the phenotype.[28,40] However, there is still no direct evidence of a causal link between these mutations and the HPFH phenotype. The one situation where a typical $\delta\beta$-thalassemia phenotype was found to exist in the absence of a detectable deletion was in the Sardinian $\delta\beta$-thalassemia.[29] The phenotype was characterized by an absence of β-globin production and an increase in the $^{A}\gamma$ type of fetal hemoglobin.[30] Pirastu et al[31] and Guida et al[32] demonstrated that the β-globin gene on this chromosome carried a nonsense mutation at the position corresponding to amino acid number 39. They suggested that this $\delta\beta$-thalassemia is likely to be the result of two independent mutations, one that silences the β-globin gene and another mutation that elevates the $^{A}\gamma$-globin production.[31,32] Recently Giglioni et al showed the presence of a point mutation at position -196 of the $^{A}\gamma$ gene in Sardinian $\delta\beta$-thalassemia. This same mutation was previously identified in the $^{A}\gamma$-gene promoter of the $^{A}\gamma$ HPFH chromosome of a Southern Italian individual.[33] Thus it seems quite likely that the Sardinian $\delta\beta$-thalassemia is the result of two independent mutations.

The $\delta\beta$-thalassemia in this Chinese family is particularly interesting. The phenotype is similar to the deletion type of $\delta\beta$-thalassemia, since both $^{G}\gamma$ and $^{A}\gamma$ genes are activated and δ and β genes are suppressed. However, extensive mapping studies failed to show any detectable deletion in the β-globin cluster extending from the ϵ gene to 20 kb downstream of the β gene. To explain the phenotype on the basis of independent mutations in the fetal and adult genes, one would have to envision four independent mutations affecting the $^{G}\gamma$,$^{A}\gamma$,δ, and β genes.

A more likely possibility would be a single mutation in a region critical for the switching from predominantly fetal globin expression to adult globin expression. Such a single mutation could explain the $\delta\beta$-thalassemia phenotype in this Chinese family and could theoretically be located either within or outside of the β-globin gene cluster. An analogous example of such a phenomenon is provided by heterocellular HPFH, a genetically heterogeneous condition associated with a much more modest elevation of HbF that is of both the $^{G}\gamma$ and $^{A}\gamma$ types.[1,4] In many cases of heterocellular HPFH, the determinant is definitely linked to the β-gene cluster,[34-37] though in some cases, perhaps at some distance from it,[34,37] whereas in others, it is clearly not linked to the cluster.[37,38] It would be very interesting to determine whether the determinant of this phenotype is linked to the β-globin cluster in this family. To do this one must study linkage of the phenotype to multiple restriction site polymorphisms in the β-globin cluster in as many individuals as possible from this family. Unfortunately, such a study is not feasible at present with only three members of this family residing in the United States. Nevertheless, the genetic locus responsible for the $\delta\beta$-thalassemia in this family may be the same as that for some cases of heterocellular HPFH, but mutated in a fashion that results in higher levels of HbF production and, concomitantly, lower levels of HbA_2 and HbA production.

Recently, a nondeletion form of HPFH associated with the synthesis of both $^{G}\gamma$ and $^{A}\gamma$ chains of HbF has been characterized in a Chinese individual.[39] However, the mutation in the latter individual is likely to be different from that of our patient because it is associated with a different haplotype of RFLPs and with a significantly higher ratio of $^{G}\gamma$ to $^{A}\gamma$ chain synthesis.

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REFERENCES

1. Weatherall DJ, Clegg JB. The Thalassemia Syndromes (ed 3) Oxford, Blackwell, 1981

2. Kinney TR, Friedman SH, Cifuentes E, Kim HC, Schwartz E: Variations in globin synthesis in delta-beta–thalassemia Br J Haematol 38.15, 1978

3. Collins FS, Weissman SM: The molecular genetics of human hemoglobin. Prog Nucleic Acid Res Mol Biol 31.315, 1984

4. Bunn HF, Forget BG: Hemoglobin: Molecular, Genetic and Clinical Aspects Philadelphia, Saunders, 1986

5 Williams JW, Beutler E, Erslev AJ, Lichtman MA: Hematology (ed 3). New York, McGraw-Hill, 1983

6. Kleihauer E, Braun H, Betke K: Demonstration von fetalen hamoglobin in den erythrozyten eines blutausstriches. Klin Wochenschr 35:637, 1957

7. Clegg JB, Naughton MA, Weatherall DJ: An improved method for the characterization of human haemoglobin mutants: Identification of $\alpha_2\beta_2{}^{95glu}$ haemoglobin N (Baltimore). Nature 207:945, 1965

8 Huisman THJ, Wilson JB. Recent advances in the quantitation of human fetal hemoglobins with different gamma chains Am J Hematol 9:255, 1980

9. Alter BP. The $^{G}\gamma$ $^{A}\gamma$ composition of fetal hemoglobin in fetuses and newborns. Blood 45:1158, 1979

10. Blin N, Stafford DW: Isolation of high molecular-weight DNA. Nucleic Acids Res 3:2303, 1976

11 Southern E: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

12 Antonarakis SE, Kazazian HH, Orkin SH: DNA polymorphism and molecular pathology of the human globin gene clusters Hum Genet 69:1, 1985

13. Kaufman RE, Kretschmer PJ, Adams JW, Coon HC, Anderson WF, Nienhuis AW: Cloning and characterization of DNA sequences surrounding the human γ-, δ-, and β-globin genes. Proc Natl Acad Sci USA 77:4229, 1980

14 Tuan D, Feingold E, Newman M, Weissman SM, Forget, BG. Different 3′ end points of deletions causing $\delta\beta$-thalassemia and hereditary persistence of fetal hemoglobin: Implications for the control of γ-globin gene expression in man Proc Natl Acad Sci USA 80.6937, 1983

15. Cheng T-C, Orkin ST, Antonarakis ST, Potter MJ, Sexton JP, Markham AF, Giardina PJV, Li A, Kazazian HH. β-thalassemia in Chinese: Use of in vivo RNA analysis and oligonucleotide hybridization in systematic characterization of molecular defects. Proc Natl Acad Sci USA 81:2821, 1984

16. Kazazian HH, Waber PG, Boehm CD, Lee JI, Antonarakis SE, Fairbanks VF. Hemoglobin E in Europeans: Further evidence for multiple origins of the β^{E}-globin gene Am J Hum Genet 36:212, 1984

17. Fritsch EF, Lawn RM, Maniatis T· Characterization of deletions which affect the expression of fetal globin genes in man. Nature 279.598, 1979

18. Tuan D, Murnane M, deRiel JK, Forget BG: Heterogeneity in the molecular basis of hereditary persistence of fetal haemoglobin. Nature 285·335, 1980

19. Ottolenghi S, Giglioni B, Comi P, Giani AM, Polli E, Acquaye CTA, Oldham JH, Masera G: Globin gene deletion in HPFH, $\delta\beta$ thalassemia and Hb Lepore disease. Nature 278:654, 1979

20. Bank A, Mears G, Ramirez F: Disorders of human hemoglobin. Science 207:486, 1980

21 Balsley JF, Rappaport E, Schwartz E, Surrey S. The γ-δ-β–globin gene region in $^{G}\gamma$-β^{+}–hereditary persistence of fetal hemoglobin. Blood 59:828, 1982

22. Huisman THJ, Shroeder WA, Efremov GD, Duma H, Mladenovski B, Brodie C, Rachmilewitz EA, Bouver N, Miller A, Brodie A, Shelton JR, Shelton JB, Apell G: The present status of the heterogeneity of fetal hemoglobin in β-thalassemia An attempt to unify some observations in thalassemia and related conditions. Ann NY Acad Sci 232:107, 1974

23. Bernards R, Flavell RA: Physical mapping of the globin gene deletion in hereditary persistence of foetal haemoglobin (HPFH). Nucleic Acid Res 8.1521, 1980

24 Feingold EA, Collins FS, Metheral JE, Stoeckert CJ Jr, Weissman SM, Forget BG: Molecular analysis of deletion and non-deletion hereditary persistence of fetal hemoglobin, in Stamatoyannopoulos G, Nienhuis AW (eds)· Experimental Approaches for the Study of Hemoglobin Switching. New York, Liss, 1985, p 107

25. Collins FS, Stoeckert CJ Jr, Serjeant GR, Forget BG, Weissman SM: $^{G}\gamma\beta^{+}$ hereditary persistence of fetal hemoglobin: Cosmid cloning and identification of a specific mutation 5′ to the $^{G}\gamma$ gene. Proc Natl Acad Sci USA 81:4894, 1984

26 Collins FS, Metherall JE, Yamakawa M, Pan J, Weissman SM, Forget BG: A point mutation in the $^{A}\gamma$-globin gene promoter in Greek hereditary persistence of fetal haemoglobin. Nature 313:325, 1985

27 Gelinas R, Endlich B, Pfeiffer C, Yagi M, Stamatoyannopoulos G: G to A substitution in the distal CCAAT box of the $^{A}\gamma$-globin gene in Greek hereditary persistence of fetal haemoglobin. Nature 313:323, 1985

28 Collins FS, Boehm CD, Waber PG, Stoeckert CJ, Weissman SM, Forget BG, Kazazian HH Concordance of a point mutation 5′ to the $^{G}\gamma$ gene with $^{G}\gamma\beta^{+}$ hereditary persistence of fetal hemoglobin in the black population Blood 64 1292, 1984

29. Ottolenghi S, Giglioni B, Taramelli R, Comi P, Mazza U, Saglio G, Camaschella C, Izzo P, Cao A, Galanello R, Gimferrer E, Baiget M, Gianni AM· Molecular comparison of $\delta\beta$-thalassemia and hereditary persistence of fetal hemoglobin DNA's: Evidence of regulatory area? Proc Natl Acad Sci USA 79:2347, 1982

30. Cao A, Melis MA, Galanello R, Angius A, Furbetta M, Giordano P, Bernini LF: $\delta\beta$(F)-thalassaemia in Sardinia. J Med Genet 19:184, 1982

31 Pirastu M, Kan YW, Galanello R, Cao A: Multiple mutations produce $\delta\beta^{o}$ thalassemia in Sardinia. Science 223:929, 1984

32. Guida S, Giglioni B, Comi P, Ottolenghi S, Camaschella C, Saglio G. The β-globin gene in Sardinian $\delta\beta^{o}$-thalassemia carries a C→T nonsense mutation at codon 39. EMBO J 3:785, 1984

33. Giglioni B, Casini C, Mantovani R, Merli S, Comi P, Ottolenghi S, Saglio G, Camaschella C, Mazza U: A molecular study of a family with Greek hereditary persistence of fetal hemoglobin and β-thalassemia. EMBO J 3:2641, 1984

34. Wood WG, Weatherall DJ, Clegg JB· Interaction of hetero-

cellular hereditary persistence of foetal haemoglobin with β thalassemia and sickle cell anaemia. Nature 264:247, 1976

35. Dover GJ, Boyer SH: F-cell production in sickle cell anemia: Regulation by genes linked to β-hemoglobin locus Science 211:1441, 1981

36. Old JM, Ayyub H, Wood WG, Clegg JB, Weatherall DJ: Linkage analysis of nondeletion hereditary persistence of fetal hemoglobin. Science 215:981, 1983

37. Gianni AM, Bregni M, Cappellini MD, Fiorelli G, Taramelli R, Giglioni B, Comi P, Ottolenghi S: A gene controlling fetal hemoglobin expression in adults is not linked to the non-α globin cluster. EMBO J 2:921, 1983

38. Giampaolo A, Mavilio F, Sposi NM, Care A, Massa A, Cianetti L, Petrini M, Russo R, Cappellini MD, Marinucci M: Heterocellular hereditary persistence of fetal hemoglobin (HPFH). Molecular mechanisms of abnormal γ-gene expression in association with β thalassemia and linkage relationship with the β-globin gene cluster Hum Genet 66:151, 1984

39. Zeng Y-T, Huang S-Z, Chen B, Liang Y-C, Chang Z-M, Harano T, Huisman THJ: Hereditary persistence of fetal hemoglobin or $(\delta\beta)^o$-thalassemia: Three types observed in south-Chinese families. Blood 66·1430, 1985

40. Waber PG, Bender MA, Gelinas RE, Kattamis C, Karaklis A, Sofroniadou K, Stamatoyannopoulos G, Collins FS, Forget BG, Kazazian HH Jr: Concordance of a point mutation 5′ to the $^A\gamma$-globin gene with $^A\gamma\beta^+$ hereditary persistence of fetal hemoglobin in Greeks. Blood 67:551, 1986.

41. Ottolenghi S, Giglioni B, Pulazzini A, Comi P, Camaschella C, Serra A, Guerrasio A, Saglio G: Sardinian $\delta\beta^o$ thalassemia. A further example of a C to T substitution at position -196 of the $^A\gamma$-globin gene promoter. Blood (in press)

Cyclophosphamide Cardiotoxicity: An Analysis of Dosing as a Risk Factor

By Mark A. Goldberg, Joseph H. Antin, Eva C. Guinan, and Joel M. Rappeport

Patients who undergo bone marrow transplantation are generally immunosuppressed with a dose of cyclophosphamide (CYA) which is usually calculated based on the patient's weight. At these high doses of CYA, serious cardiotoxicity may occur, but definitive risk factors for the development of such cardiotoxicity have not been described. Since chemotherapeutic agent toxicity generally correlates with dose per body surface area, we retrospectively calculated the dose of CYA in patients transplanted at our institution to determine whether the incidence of CYA cardiotoxicity correlated with the dose per body surface area. Eighty patients who were to receive CYA 50 mg/kg/d for four days as preparation for marrow grafting underwent a total of 84 transplants for aplastic anemia, Wiskott-Aldrich syndrome, or severe combined immunodeficiency syndrome. Fourteen of 84 (17%) patients had symptoms and signs consistent with CYA cardiotoxicity within ten days of receiving 1 to 4 doses of CYA. Six of the 14 patients died with congestive heart failure. The dose of CYA per body surface area was calculated for all patients and the patients were divided into two groups based on daily CYA dose: Group 1, CYA $\leq 1.55\ \mathrm{g/m^2/d}$; Group 2, CYA $> 1.55\ \mathrm{g/m^2/d}$. Cardiotoxicity that was thought to be related to CYA occurred in 1/32 (3%) of patients in Group 1 and in 13/52 (25%) patients in Group 2 ($P < 0.025$). Congestive heart failure caused or contributed to death in 0/32 patients in Group 1 v 6/52 (12%) of patients in Group 2 ($P < 0.25$). There was no difference in the rate of engraftment of evaluable patients in the two groups ($P > 0.5$). We conclude that the CYA cardiotoxicity correlates with CYA dosage as calculated by body surface area, and that patients with aplastic anemia and immunodeficiencies can be effectively prepared for bone marrow grafting at a CYA dose of $1.55\ \mathrm{g/m^2/d}$ for four days with a lower incidence of cardiotoxicity than patients whose CYA dosage is calculated based on weight. This study reaffirms the principle that drug toxicity correlates with dose per body surface area.

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IN order to achieve successful bone marrow engraftment, effective immunosuppression of the bone marrow recipient is of paramount importance. Cyclophosphamide (CYA) is an alkylating agent that has been shown to have both potent immunosuppressive properties and antineoplastic activity,[1] and it is a mainstay in most pretransplant preparative regimens. In 1970, Storb and colleagues investigated the hematologic and pathologic effects of various high-dose CYA regimens in rhesus monkeys. Monkeys given 240 mg/kg of CYA over one to four days had a greater than 75% incidence of cardiotoxicity.[2] Subsequently, in 1971, Santos et al reported the first case of fatal CYA cardiotoxicity in a human.[3] Since 1971, two series and numerous case reports have appeared in the literature, indicating that cardiotoxicity can occur in patients receiving 120 to 270 mg/kg of CYA over one to eight days. These patients developed severe and often fatal congestive heart failure soon after the first dose of CYA.[4-14] Unfortunately, it is difficult to determine the true incidence and contribution of CYA to the cardiotoxicity since the literature contains primarily case reports and the CYA was administered in various dosages and often with other potentially cardiotoxic agents. However, if the series of Steinherz et al[13] and Gottdiener et al[14] are combined, the incidence of symptomatic cardiomyopathy is 22% (16/72) and fatal cardiotoxicity is 11% (8/72).

It is noteworthy that there are no case reports of fatal cardiotoxicity in humans less than 12 years old, suggesting that there is either an intrinsic resistance to the cardiotoxic effects of CYA in children or that children received a lower relative dose of CYA. In most bone marrow transplant protocols CYA is administered on a mg/kg basis, even though chemotherapeutic agent toxicity in general[15,16] and CYA toxicity in particular[16] have been shown to correlate with dose per body surface area. We retrospectively analyzed the occurrence of cardiotoxicity in patients with aplastic anemia and immunodeficiency syndromes treated at our institutions over the last 13 years. We chose patients with aplastic anemia or immunodeficiency syndromes because we used the same dosage of CYA to prepare patients with these diseases for marrow transplantation, and we avoided the confounding effects of prior anthracycline use and total body irradiation which are used in patients with malignancies. This article summarizes our analysis of CYA dosing and cardiotoxicity in 80 patients undergoing a total of 84 marrow transplants.

METHODS

In accordance with institutional review board guidelines, we reviewed the records of 80 consecutive patients with aplastic anemia, severe combined immune deficiency syndrome (SCIDS), and the Wiskott-Aldrich syndrome (WAS) who received high-dose CYA as part of the preparative regimen for bone marrow transplantation between 1972 and 1985 at the Brigham and Women's Hospital and the Children's Hospital Medical Center in Boston. Patients who received total body irradiation or who had known prior heart disease were excluded from the analysis. The only patients with SCIDS who were prepared with CYA received T cell–depleted marrow inocula

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from haploidentical family members. These patients received or were scheduled to receive CYA at a dosage of 50 mg/kg/d for four consecutive days. Other immunosuppressive and myelotoxic agents are listed in Table 1; these regimens have been reported previously.[17–20] In brief, 49 patients with aplastic anemia received rabbit antithymocyte serum (ATS) 0.2 mL/kg every other day for 3 doses, procarbazine 12.5 mg/kg every other day for 3 doses, and CYA 50 mg/kg/d for four days; 15 patients were prepared with CYA 50 mg/kg/d for four days alone, and one patient with aplastic anemia complicating paroxysmal nocturnal hemoglobinuria received busulfan 16 mg/kg in divided doses in addition to the CYA, ATS, and procarbazine Children with SCIDS receiving haploidentical, T cell–depleted marrow transplants were treated similarly to patients with aplastic anemia except they did not receive the procarbazine.[19] The patients with WAS received ATS, CYA 50 mg/kg/d for four days, and busulfan 8 to 16 mg/kg in divided doses. All but one patient with aplastic anemia were transplanted from HLA identical, MLR compatible siblings; the only exception was a patient with a D/DR crossover, and thus, a single antigen mismatch. Patients with WAS were transplanted from either HLA identical or haploidentical relatives and all haploidentical transplants were performed with T cell–depleted marrow (Table 1).[18,19]

Cyclophosphamide cardiotoxicity was defined as the occurrence of symptomatic congestive heart failure within ten days of receiving the first dose of CYA. All such patients were followed with frequent chest x rays and electrocardiograms. Eight of 14 symptomatic patients had M-mode or real time echocardiograms. Routine posttransplant echocardiograms were not obtained.

Engraftment was assessed by the recovery of granulocytes to >500 cells/μL, platelets >20,000 cells/μL without platelet transfusion, reticulocytes >1%, red blood cell typing for 30 RBC antigens, cytogenetics, DNA polymorphism analysis, and blastogenic response to lectins as described previously.[17–20]

Statistical methods. χ^2 analysis and Student's *t* test were performed with the Systat statistical software package (Evanston, Ill), and the life-table analysis and log-rank test were performed as described by Peto et al.[21] All χ^2 analyses utilized the Yates continuity correction

RESULTS

Patient characteristics. The age, sex, diagnoses, HLA compatibility, and preparative regimens are shown in Table 1. Eighty patients underwent 84 evaluable transplants (2 patients with aplastic anemia and 2 patients with SCIDS each received 2 transplants). Sixty-three patients had severe aplastic anemia, 10 patients had SCIDS, and 7 patients had WAS.

Four of the patients had aplastic anemia as a complication of paroxysmal nocturnal hemoglobinuria and none had Fanconi's anemia. Because of the nature of the diseases, patients with WAS and SCIDS were significantly younger than the patients with aplastic anemia (Student's *t* test, $P < 0.01$).

Incidence of cyclophosphamide cardiotoxicity. In 14/84 (17%) transplants, signs and symptoms consistent with congestive heart failure developed within ten days of receiving the first dose of CYA. The clinical diagnosis of congestive heart failure was supported by echocardiography in 6 patients and was equivocal in 2. Six of the 14 (43%) died with congestive heart failure, although in 4/6 sepsis may have contributed. Postmortem examinations were obtained in 4 of the 6 patients and findings consistent with CYA cardiotoxicity[8] were present in all four. In none of the cases that came to autopsy was there evidence of cardiac hemosiderosis. All of the cases of congestive heart failure were found in the group with aplastic anemia (14/65 [22%]); no patients with SCIDS or WAS developed clinical cardiotoxicity (0/19).

Correlation of age and dose per body surface area with the occurrence of cardiotoxicity. Patients with cardiotoxicity were significantly older than patients without clinically apparent cardiac injury (median ages 18 years *v* 2 years; Student's *t* test, $P < 0.01$). When cardiotoxicity did occur in children it was not fatal. Since small patients have a greater relative body surface area, we recalculated the dose of CYA that they received based on body surface area. The dose of CYA was 1.822 ± 0.203 g/m^2/d (mean ± SD) in patients with cardiotoxicity and $1.532 \pm .380$ g/m^2/d (mean ± SD) in patients without cardiac toxicity (Student's *t* test, $P < 0.005$). The relationship of age and dose/m^2 is shown in Fig 1. It is clear that children received much lower relative doses of CYA than did adults and adolescents. Because some patients developed cardiotoxicity before the entire dose of 200 mg/kg was administered, data were analyzed based on daily dosage rather than total dosage. The relationship between cardiotoxicity and dose per body surface area is shown in Fig 2. Since there was a dose of CYA below which it

Table 1. Clinical Characteristics

Diagnosis	n	Median Age, yrs (range)	Sex (M/F)	Degree of HLA Compatibility	Preparative Regimens*
Aplastic anemia	63	18 (2–52)	38/25	62 HLA match	ATS/CYA/PC (48) CYA (15) Bu/ATS/CYA/PC (1)
				1 mismatch	ATS/CYA/PC (1)
Wiskott-Aldrich Syndrome	7	2.75 (0.75–5 75)	7/0	5 HLA match	Bu/ATS/CYA (4) Ara-C/CYA (1)
				2 mismatch	Bu/ATS/CYA (2)
SCIDS	10	0.75 (0.33–1.5)	6/4	10 mismatch	ATS/CYA (9) Bu/ATS/CYA (2) ATS/CYA/PC (1)
Total	80				

Abbrev: Ara-C, arabinosyl cytosine, ATS, antithymocyte serum, Bu, busulfan; CYA, cyclophosphamide; PC, procarbazine; SCIDS, severe combined immunodeficiency syndrome

*Numbers in parentheses indicate the number of transplants done with the indicated regimen. Two patients with aplastic anemia and two patients with SCIDS were transplanted twice.

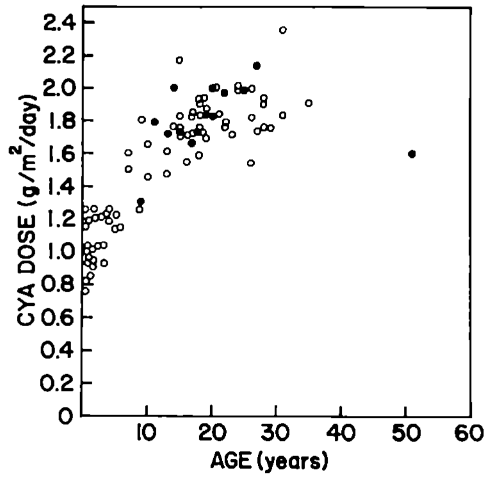

Fig 1. Daily dose of CYA per body surface area expressed as a function of age. All of these patients received CYA doses calculated on the basis of weight; the doses per body surface area were then retrospectively determined. Open circles, no cardiotoxicity; closed circles, cardiotoxicity.

appeared unlikely that a subject would develop cardiotoxicity, the patients were divided in two groups based on daily CYA dose. Group 1 received CYA ≤ 1.55 g/m^2/d and Group 2 received CYA >1.55 g/m^2/d. The median age of the patients in Group 1 was two years (range 4/12 to 26 years) while the median age in Group 2 was 18 years (range 17 to 52 years) and this difference was statistically significant (Student's *t* test, $P < 0.01$). Cardiotoxicity occurred in 1/32 (3%) patients in Group 1 compared to 13/52 (25%) of patients in Group 2 ($\chi^2 = 5.34$, $P < 0.025$). When the patients with aplastic anemia were analyzed separately, cardiotoxicity occurred predominantly in the patients in Group 2 (13/52 *v* 1/13, $\chi^2 = 0.85$, $P > 0.25$). Although the analysis did not reach statistical significance, the trend suggests that the difference would have been significant if more patients were studied.

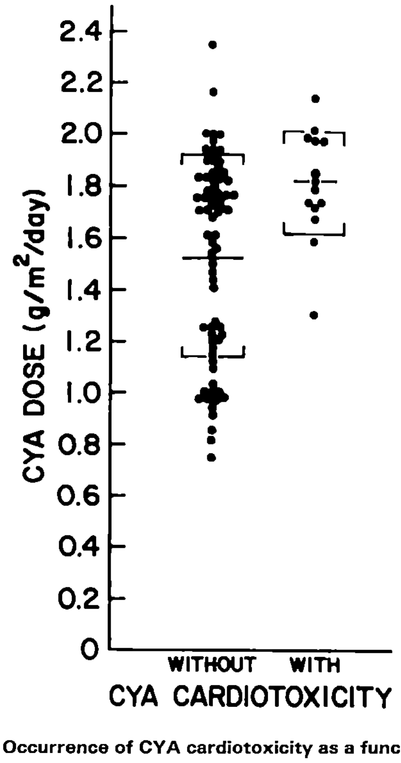

Fig 2. Occurrence of CYA cardiotoxicity as a function of daily dose per body surface area. Bars indicate mean ± SD.

It is important to note that we did not observe cumulative CYA cardiotoxicity in the 4 subjects who received more than one transplant and therefore received CYA 400 mg/kg total dose. However, it is possible that cumulative toxicity might be apparent if more patients are studied. Congestive heart failure caused or contributed to death in 0/32 patients in Group 1 *v* 6/52 (12%) of patients in Group 2 ($\chi^2 = 2.43$, $P < 0.25$).

Engraftment. Patients were considered evaluable for determination of engraftment if they lived for at least 21 days after transplant. Since the patients with SCIDS received haploidentical transplants and were likely to develop only lymphoid grafts, and the children with WAS received busulfan, we only analyzed the evaluable individuals with aplastic anemia in Groups 1 and 2. In Group 1, 9/10 (90%) of the patients engrafted compared with 33/41 (81%) of the patients in Group 2 ($\chi^2 = 0.06$, $P > 0.5$). There was also no difference in survival when assessed by life-table analysis and the log-rank test ($\chi^2 = 1.012$, $P > 0.5$). It is possible that differences in engraftment would be apparent if more patients were studied; however, our data do not suggest a trend toward a higher rate of engraftment with higher doses of CYA.

DISCUSSION

In the early 1970s Santos and coworkers[3,4] and the Seattle group[8] pioneered the use of cyclophosphamide in the immunosuppression of patients in preparation for bone marrow grafting. Cyclophosphamide was generally administered in a total dose of 200 mg/kg for patients with aplastic anemia and 120 mg/kg for patients with malignancies who would also receive total body irradiation. Based on those early studies most transplant units, including ours, employed similar preparative regimens. It became gradually clear that this dose of cyclophosphamide could cause a lethal cardiotoxicity characterized by severe, often refractory, congestive heart failure occurring one to ten days after the administration of the first dose of cyclophosphamide.[3-14] The clinical syndrome of severe congestive heart failure was accompanied by electrocardiographic findings of diffuse voltage loss, cardiomegaly, pulmonary vascular congestion, and pleural effusions on chest radiograph, and echocardiographic demonstration of decreased fractional ventricular wall shortening, increased end-diastolic volume, and pericardial effusions. Pathological examination of the heart reveals hemorrhagic myocardial necrosis, thickening of the left ventricular wall, serosanguinous pericardial effusions, and fibrinous pericarditis. Electron microscopy has shown multifocal myocardial necrosis associated with fibrin microthrombi near areas of capillary endothelial damage. These findings are thought to be consistent with myocardial necrosis following cyclophosphamide-induced endothelial damage.[8]

No definitive risk factors for the development of CYA cardiotoxicity have yet been identified. It does not seem to be related to the underlying disease or anemia or thrombocytopenia.[13] It is possible that prior anthracycline chemotherapy and/or radiation therapy may predispose to the development of CYA cardiotoxicity.[13] However, we excluded from evaluation all patients who had received anthracyclines or any radiation therapy, and in the absence of any demonstrable prior cardiac dysfunction it is likely that the cardiomyopathy observed in this series is due to CYA alone. Although procarbazine is not known to be associated with cardiotoxicity, fewer children received it as part of the preparatory regimen and we cannot exclude the possibility that procarbazine can contribute to the cardiotoxicity of CYA. Clearly, sepsis can be associated with cardiac dysfunction,[22] and since 4/6 patients who died were septic, it is likely that sepsis contributed to the severity of the cardiac damage. Cardiac injury due to transfusion-associated iron overload could conceivably predispose patients to CYA-induced cardiotoxicity; however, none of our patients who died had iron deposition in the heart, and most patients with aplastic anemia who have an acceptable donor are transplanted before they have received the 50 or more units of red blood cells required to produce hemosiderosis.

The rationale for using CYA at a dosage of 200 mg/kg is based both on prior animal studies and the observation that 180 mg/kg used alone to prepare patients with acute lymphoblastic leukemia for marrow transplant resulted in a high relapse rate.[23-26] However, the extrapolation of animal studies to humans is often difficult. In 1958, Pinkel et al showed that the effects of chemotherapeutic agents on animals and humans were similar when the dose was calculated as dose per unit of body surface area, but quite dissimilar when calculated per unit of weight.[15] Freireich and colleagues subsequently made a quantitative comparison of the toxicity of multiple anticancer agents including CYA in the mouse, rat, hamster, dog, monkey, and man. They also observed that the toxicities were quite similar between species when dose was calculated based on body surface area.[16]

We observed an extremely low incidence of CYA cardiotoxicity in our pediatric patients. Since children have a relatively smaller ratio of weight to body surface area than do adults, we hypothesized that the children were actually receiving relatively less CYA than the adult patients. Furthermore, by similar analysis obese patients were likely to be overdosed when treated on a mg/kg basis This suspicion was confirmed when we calculated the CYA dose based on body surface area. Thus, the dose of CYA administered ranged from 0.75 mg/m^2/d to 2.35 mg/m^2/d, and this range was due largely to age related difference in body surface area (Fig 1). Cardiotoxicity was significantly more likely to occur at a CYA dose of >1.55 g/m^2/d (Fig 2). However, it did not appear that the lower dose compromised the transplant. There was no dose-related difference in the rate of engraftment or survival between the two groups. In this retrospective analysis, it can be argued that the lower incidence of cardiotoxicity in the younger patients is due to an intrinsic, age-related difference in the susceptibility to CYA-induced heart damage. However, the data presented cannot address that concern since inferences about relative susceptibility to drug effects can only be made if the dosing is the same.

This analysis suggests that cyclophosphamide cardiotoxicity is dose related and that patients with aplastic anemia, SCIDS, and WAS can be effectively prepared for marrow grafting with CYA 1.55 g/m^2/d for 4 consecutive doses with equivalent likelihood of engraftment and less cyclophosphamide cardiotoxicity. It is likely that a similar change in dosing would reduce the incidence of cardiotoxicity in patients transplanted for malignancies.

REFERENCES

1. Gershwin ME, Goetzl EJ, Steinberg AD: Cyclophosphamide: Use in practice. Ann Intern Med 80.531, 1974

2. Storb R, Buckner CD, Dillingham LA, Thomas ED: Cyclophosphamide regimens in rhesus monkeys with and without marrow infusion. Cancer Res 30:2195, 1970

3 Santos GW, Sensenbrenner LL, Burke PJ, Colvin M, Owens AH Jr, Bias WB, Slavin RE· Marrow transplantation in man following cyclophosphamide Transplant Proc 3:400, 1971

4. Santos GW, Sensenbrenner LL, Burke PJ, Mullins GM, Bias WB, Tutschka PJ, Slavin RE: Use of cyclophosphamide for clinical marrow transplantation. Transplant Proc 4:559, 1972

5. Buckner CD, Rudolph RH, Fefer A, Clift RA, Epstein RB, Funk DD, Neiman PE, Slichter SJ, Storb R, Thomas ED: High-dose cyclophosphamide therapy for malignant disease. Cancer 29:357, 1972

6. Buckner CD, Clift RA, Fefer A, Funk DD, Glucksberg H, Ramberg RE, Storb R, Thomas ED: Aplastic anemia treated by marrow transplantation. Transplant Proc 5.913, 1973

7 Mullins GM, Anderson PN, Santos GW: High dose cyclophosphamide therapy in solid tumors. Cancer 36:1950, 1975

8. Buja LM, Ferrans VJ, Graw RG Jr: Cardiac pathologic findings in patients treated with bone marrow transplantation. Human Pathol 7:17, 1976

9. Appelbaum FR, Strauchen JA, Graw RG Jr, Savage DD, Kent KM, Ferrans VJ, Herzig GP: Acute lethal carditis caused by high-dose combination chemotherapy Lancet i:58, 1976

10 Appelbaum FR, Deisseroth AB, Graw RG Jr, Herzig GP, Levine AS, Magrath IT, Pizzo PA, Poplack DG, Ziegler JL: Prolonged complete remission following high dose chemotherapy of Burkitt's lymphoma in relapse. Cancer 41·1059, 1978

11. Mills BA, Roberts RW· Cyclophosphamide-induced cardiomyopathy. Cancer 43:2223, 1979

12. von Bermuth G, Adam D, Hofstetter R, Lang D, Kohne E, Niethammer D: Cyclophosphamide cardiotoxicity. Eur J Pediatr 134:87, 1980

13 Steinherz LJ, Steinherz PG, Mangiacasale D, O'Reilly R, Allen J, Sorell M, Miller DR: Cardiac changes with cyclophosphamide. Med Pediatr Oncol 9:417, 1981

14 Gottdiener JS, Appelbaum FR, Ferrans VJ, Deisseroth A, Zeigler J: Cardiotoxicity associated with high-dose cyclophosphamide therapy. Arch Intern Med 141·768, 1981

15. Pinkel D: Use of body surface area as a criterion of drug dosage in cancer chemotherapy Cancer Res 18:853, 1958

16. Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE: Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. Cancer Chemother Rep 50.219, 1966

17 Smith BR, Guinan EC, Parkman R, Ferrara J, Levey RH,

Nathan DG, Rappeport JM: Efficacy of a cyclophosphamide-procarbazine-antithymocyte serum regimen for prevention of graft rejection following bone marrow transplantation for transfused patients with aplastic anemia. Transplantation 39:671, 1985

18. Parkman R, Rappeport JM, Hellman S, Lipton JM, Smith B, Gehan R, Nathan DG: Busulfan and total body irradiation as antihematopoietic stem cell agents in the preparation of patients with congenital bone marrow disorders for allogeneic bone marrow transplantation Blood 64:852, 1984

19. Reinherz EL, Geha R, Rappeport JM, Wilson M, Penta AC, Hussey RE, Fitzgerald KA, Daley JF, Levine H, Rosen FS, Schlossman SF: Reconstitution after transplantation with T-lymphocyte-depleted HLA haplotype-mismatched bone marrow for severe combined immunodeficiency. Proc Natl Acad Sci USA 79:6047, 1982

20. Ginsburg D, Antin JH, Smith BR, Orkin SH, Rappeport JM: Origin of cell populations after bone marrow transplantation. Analysis using DNA sequence polymorphisms. J Clin Invest 75:596, 1985

21. Peto R, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, Mantel N, McPherson K, Peto J, Smith PG: Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples Br J Cancer 35·1, 1977

22. Parker MM, Shelhamer JH, Bacharach SL, Green MV, Natanson C, Frederick TM, Damske BA, Parillo JE: Profound but reversible myocardial depression in patients with septic shock. Ann Intern Med 100:483, 1984

23. Thomas ED, Buckner CD, Banaji M, Clift RA, Fefer A, Flournoy N, Goodell BW, Hickman RO, Lerner KG, Neiman PE, Sale GE, Sanders JE, Singer J, Stevens M, Weiden PL: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood 49:511, 1977

24. Santos GW: Application of marrow grafts in human disease· Its problems and potential, in Hanna MG Jr (ed): Contemporary Topics in Immunobiology (vol 1). New York, Plenum, 1972, p 143

25. Santos GW: History of bone marrow transplantation. Clin Haematol 12:611, 1983

26. Graw RG Jr, Leventhal BG, Yankee RA, Rogentine GN, Whang-Peng J, Ginnif MH, Herzig GP, Halterman RH, Henderson ES: HLA and mixed leukocyte culture matched allogeneic bone marrow transplantation in patients with acute leukemia. Transplant Proc 3:405, 1971

Low-Dose Deoxycoformycin in the Treatment of Hairy Cell Leukemia

By Eric H. Kraut, Bertha A. Bouroncle, and Michael R. Grever

Ten patients with progressive hairy cell leukemia were treated with 2′deoxycoformycin (dCF) by intravenous bolus (4 mg/m²) given every other week. All ten patients are evaluable for response and nine of the ten patients have achieved a complete remission. In addition to clearing of hairy cells from the bone marrow, eight patients had resolution of their monocytopenia. Seven of the nine patients remain in unmaintained remission with a median duration of 6.2 months. Two patients have had relapse in the bone marrow alone and continue to have normal peripheral blood counts. They are being followed without treatment. Toxicity was minimal at this low dose with one patient having a mild reversible reduction in creatinine clearance. Four other patients had reversible neutropenia. There were no significant infections associated with treatment. Low-dose deoxycoformycin administered intravenously every other week represents an extremely effective treatment for hairy cell leukemia.

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HAIRY cell leukemia was first described at the Ohio State University by Bouroncle, Wiseman, and Doan in 1958, as a new hematologic and pathologic entity.[1] It is a chronic form of leukemia usually manifested by splenomegaly, pancytopenia, and the presence in the blood and bone marrow of the pathognomonic hairy cell, a mononuclear cell with cytoplasmic projections.[2] Present evidence suggests that this is a lymphoproliferative disease that like chronic lymphocytic leukemia originates most frequently in the B lymphocyte line.[3-5] The clinical course is variable with some patients remaining asymptomatic for months to years, while others require treatment for symptoms related to hypersplenism, recurrent infection, or bone marrow failure.[6,7]

The standard treatment for these patients has been splenectomy. Although splenectomy may improve the hematologic complications of this disease, it may not prolong survival.[2,8,9] Moreover, a significant number of patients do not respond or relapse after splenectomy. Attempts have been made to develop other effective treatment for hairy cell leukemia. Low-dose chlorambucil has offered palliation in some patients but does not completely reverse the pancytopenia or markedly alter the bone marrow infiltration.[10,11] Intensive chemotherapy has been used successfully on only a few patients, and has been associated with significant morbidity and mortality.[12,13]

Bone marrow transplantation has been accomplished in one patient with hairy cell leukemia,[14] but the associated risk and age limitations make it unfeasible in most patients. Several studies of alpha interferon in patients with this disease[15-18] have demonstrated response rates of 43% to 94% with hematologic improvement and clearing of hairy cells from the bone marrow. It is too early, however, to determine the effect of alpha interferon on survival.

In 1980, we began a study of the experimental drug 2′deoxycoformycin (dCF) in patients with lymphoproliferative malignancies.[19] This agent is a potent inhibitor of adenosine deaminase, which is an important enzyme in purine catabolism. During the course of our early investigation, we demonstrated activity of this drug in a patient with refractory hairy cell leukemia. Therefore, we initiated a specific phase II study of dCF in patients with hairy cell leukemia, the results of which are reported here.

MATERIALS AND METHODS

In May 1984, we began the study of low-dose dCF 4 mg/m² administered intravenously every other week in patients with hairy cell leukemia Informed consent approved by the Ohio State University Human Subjects Review Board was obtained before administration of Deoxycoformycin. 2′Deoxycoformycin was obtained from the Investigational Drug Branch National Cancer Institute (NCI), Bethesda, Md. The eligibility criteria for entrance to the study included the following: (1) A confirmed diagnosis of hairy cell leukemia on the basis of clinical characteristics and demonstration of hairy cells in the peripheral blood and bone marrow examination. (2) Progressive disease as demonstrated by anemia (hemoglobin <12 g/dL, thrombocytopenia platelets <100,000/μL), neutropenia (neutrophils <1,500 μL), and/or leukemia (white blood cell count >10,000/μL with more than 50% hairy cells. (3) Patients with normal hepatic and renal function with a creatinine clearance >50 mL/min.

Six of our ten patients had been splenectomized However, splenectomy was not a requirement before receiving a trial of dCF The nonsplenectomized patients had either refused this operation or were not considered to be good surgical candidates. Patients received dCF at a dose of 4 mg/m² by intravenous bolus injection every other week. Patients were carefully monitored for a change in clinical status by physical examination and peripheral blood count with a differential prior to each treatment. Treatment was modified in the face of a significant change in renal function or neutropenia according to the following guidelines. A serum creatinine was determined on each patient in the outpatient clinic immediately before each treatment If the value had increased by more than 20% over the baseline value, the dose was held until a 24-hour urine for creatinine clearance was obtained. The drug was administered only when the creatinine clearance was 50 mL/min or better. If the white blood cell count had decreased below 1,500/μL after drug administration, then the drug was held until recovery or bone marrow evaluation demonstrated that this reduction was secondary to disease.

Patients were seen every other week and response assessment was made after a minimum of two courses of therapy Criteria for response were defined as follows: (1) Complete remission (CR) The

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absence of hairy cells in the bone marrow aspirate and biopsy with restoration of the peripheral blood counts to the following values: Hemoglobin >12 g/dL, platelets >100,000/μL, and neutrophils >1,500/μL. (2) Partial remission (PR). Absence of hairy cells in the peripheral blood and a 50% or greater reduction in the percentage of hairy cell infiltration in the bone marrow biopsy, and improvement in peripheral counts as noted under CR. (3) Minor Response (MR): Improvement in one or more of the peripheral blood elements as defined above, or greater than 50% reduction in circulating hairy cells.

Patients who had obtained a complete remission were followed off treatment with duration of response measured from the time that the remission was documented.

Adenosine deaminase assay Determination of the ADA activity of the peripheral blood buffy coat preparation was performed before and one-half hour following the intravenous administration of dCF to document that enzyme inhibition in circulating leukocytes occurred in vivo. The buffy coat preparation was obtained by dextran sedimentation from heparinized blood. The cells were washed twice in Seligmann's balanced salt solution with 1% EDTA, resuspended in 5 mmol/L Tris-HCl (pH 7.4) and 0.25 mol/L sucrose, and counted. After sonication for 30 seconds, the homogenate was centrifuged at 50,000 x g and 4°C for 30 minutes. Supernatant ADA was assayed in triplicate.

The enzyme assay was carried out in a total volume of 70 μL containing 5 mmol/L Tris-HCl (pH 7.4) and 4 mmol/L adenosine at 37 °C. Radiolabeled 8-^{14}C-adenosine (ICN Pharmaceuticals, Inc, Irvine, Calif), 54 m Ci/mmol, was added to the cold substrate to detect product conversion. After ten minutes, the assay was terminated by adding 20 μL of 4 mol/L formic acid and placing the sample in an ice bath. A 5-μL aliquot was placed on a cellulose acetate thin-layer chromatography plate (Analabs, Inc, North Haven, Conn) and developed in water for 45 minutes. The addition of unlabeled markers (adenosine and inosine) at the origin permitted identification of both substrate and product migration bands for scraping and subsequent counting in a scintillation count. The ADA activity was expressed as micromoles of inosine formed per hour per 10^6 leukocytes.

Since there was a variable percentage of circulating hairy cells present in the peripheral blood of each patient, an ADA assay was also performed on a mononuclear cell preparation of the peripheral blood before dCF administration to provide an estimate of the enzyme activity in a preparation enriched for the hairy cells. The mononuclear cell pereparation was obtained from heparinized peripheral blood subjected to a Ficoll-hypaque (Pharmacia, Piscataway, NJ) separation. The cells were washed with Seligmann's balanced salt solution with 1% EDTA, resuspended in 5 mmol/L Tris-HCl (pH 7.4) and 0.25 mol/L sucrose, and counted. The mononuclear cells were sonicated on ice for 60 seconds. The homogenate was centrifuged at 50,000 x g at 4 °C for 30 minutes. Supernatant ADA was assayed as previously described.[20]

RESULTS

The characteristics of the ten patients are shown in Table 1. The mean age was 53 years (range 37 to 70) and nine of the patients were male. Six of the ten patients had previously been splenectomized and the median time to treatment after splenectomy was 28 months (range 6 to 67 months). Three patients had received chemotherapy in addition to splenectomy, either chlorambucil or 6 mercaptopurine. None of the patients were treated with interferon. The initial peripheral blood counts prior to treatment are demonstrated in Table 2. Five patients were granulocytopenic, seven patients were anemic, and seven patients were thrombocytopenic. All patients had hypercellular bone marrow biopsies with diffuse infiltration with hairy cells.

Table 1. Patient Characteristics

Patient No	Sex/Age	Prior Splenectomy	Prior Chemotherapy
1	M/51	+	—
2	M/42	+	Leukeran
3	M/62	−	—
4	M/37	+	Leukeran
5	M/70	−	—
6	M/42	+	—
7	M/42	−	—
8	F/65	+	6 MP
9	M/59	+	—
10	M/58	−	—

Abbreviation. 6MP, 6 mercaptopurine.

All ten patients are evaluable for response and toxicity. Nine of the ten patients have obtained a documented complete remission demonstrated by normalization of blood counts (Table 2) as well as clearing of hairy cells from the bone marrow. The average number of courses to complete remission was six (range 4 to 13). Seven of the nine patients that achieved complete remission remain in remission without maintenance therapy from 1 to 10 months. Two patients (Nos. 1 and 3) have had bone marrow relapse only at 12 and 8 months, respectively. Their peripheral blood counts remain normal and they are being followed without treatment until deterioration of peripheral blood counts occur. In contrast to the difficulty obtaining a bone marrow aspirate before treatment with dCF, eight of nine patients achieving a complete remission were able to be aspirated. In addition, eight patients who were severely monocytopenic prior to treatment (<100 monocytes/μL) had a demonstrable increase in the peripheral blood monocyte counts (250 to 1,080 monocytes/μL).

One of the patients who entered into complete remission had been the first patient entered on protocol and his course was quite instructive. He had originally reached a partial remission after only two doses of dCF. Further treatment was held when his creatinine clearance dropped from 55 to 43 cc/min, although this later returned to baseline level. He was followed off therapy for 9 months until there was evidence of disease progression. His therapy was restarted at 75% of the initial dose to avoid renal toxicity. He tolerated the dCF reasonably well and obtained a complete remission after 12 doses of dCF.

The only patient who has not achieved complete remission status started out with a high peripheral white blood cell count of 159,000/μL, consisting of predominantly hairy cells and has slowly responded with reduction in the peripheral blood count to 20,000.

Toxicity with this low-dose regimen was mild. Most patients reported mild to moderate nausea lasting for less than 24 hours controlled by antiemetics. Only one patient had a reversible reduction in creatinine clearance. Four patients had therapy temporarily held due to reversible neutropenia. One patient developed an inflammation of

Table 2. Hematologic Values and Response in Patients with Hairy Cell Leukemia

			Before Deoxycoformycin					After Deoxycoformycin				
Patient No	Response	Duration of Response (mo)	WBC $10^3/\mu L$	Hairy Cells (%)	Neutrophils (%)	Hgb (g/dL)	Platelets $10^3/\mu L$	WBC $10^3/\mu L$	Hairy Cells (%)	Neutrophils (%)	Hgb (g/dL)	Platelets $10^3/\mu L$
1	CR	12	15.6	72	24	12.5	266.0	8.3	0	68	15.3	330.0
2	CR	10+	16.3	65	21	11.5	186.0	5.7	0	86	14.1	314.0
3	CR	8	1.6	45	12	13.6	93.0	5.7	0	92	15.1	119.0
4	CR	7+	25.2	90	1	12.3	100.0	6.4	0	82	16.5	352.0
5	CR	3.5	1.5	4	20	10.5	34.0	4.2	0	74	13.2	237.0
6	CR	4+	2.9	10	14	7.8	67.0	10.4	0	90	14.4	523.0
7	CR	6+	1.0	89	1	8.4	94.0	6.2	0	86	15.0	196.0
8	IMP	—	159.0	92	2	15.2	141.0	20.6	61	31	16.6	292.0
9	CR	3+	23.7	98	2	11.2	110.0	6.0	0	64	13.1	274.0
10	CR	1+	1.1	4	16	9.9	90.0	4.1	0	82	12.8	183.0

Abbreviations: CR, complete remission; IMP, improvement.

multiple solar dermatosis. This resolved when the drug was held, and the patient had no further problems. No significant infectious complications developed related to dCF treatment. One patient who had been hospitalized several times for treatment of infection secondary to an eosophageal fistula and neutropenia developed pneumonia 2 weeks after dCF was initiated. This resolved on antibiotics and appeared to be a complication of his underlying problem and not secondary to the drug. One patient developed a fever within 24 hours after receiving the drug, and it resolved within 48 hours.

Adenosine deaminase activity was measured on buffy-coat preparations and peripheral blood mononuclear cell preparations when cell numbers allowed. Values differed significantly between the buffy-coat and the mononuclear cell preparation in each individual, and there was also a wide range of values among individuals (Table 3). Significant inhibition of ADA activity was achieved in all but one patient during the first course of therapy, but in subsequent courses inhibition was achieved in this patient. There was no correlation between ADA values and response to therapy or time to response.

DISCUSSION

Our results demonstrate that low-dose dCF is a highly effective treatment for patients with hairy cell leukemia. Nine of ten evaluable patients have obtained a complete hematologic remission with minimal toxicity. Although our study involves a limited number of patients, the response rate of 90% is equal to the best results achieved with alpha interferon.[15–18] Complete remissions in this disease following treatment with dCF have also been reported by two other groups using this drug with a slightly different dose and schedule.[21–24] Complete remissions were associated with clearing of hairy cells from the peripheral blood and bone marrow, clearance of bone marrow fibrosis, and ability to aspirate marrow. We also observed reversal of the severe monocytopenia characteristic of hairy cell leukemia.

Table 3. Adenosine Deaminase Activity (ADA)

Patient No	Peripheral Blood Buffy-Coat Preparation Before dCF	Peripheral Blood Buffy-Coat After dCF	Peripheral Blood Mononuclear Cell Preparation
1	4.3	ND	1.7
2	11.8	0.9	ND
4	2.6	0.5	10.4
5	7.1	0	12.5
6	5.7	0	12.4
8	0.1	0	ND

Adenosine deaminase activity was determined as described in Materials and Methods. ADA activity is expressed as 10^{-2} umol inosine formed each hour per 10^6 cells.

Seven of the nine patients remain in unmaintained remission for up to 10 months. Two patients have had early relapse with recurrence of hairy cells in the bone marrow but no change in their peripheral blood counts. Since they are doing well clinically, they are being followed carefully without treatment until further evidence of disease progression occurs. This decision was based on experience obtained from studies on the natural history of this disease[2,7,8] which suggests that disease progression may be slow and normalization of peripheral blood counts may be sufficient to prolong survival. Furthermore our experience with one patient who initially progressed off therapy and then subsequently responded to reinstitution of dCF suggests that patients can be successfully retreated. Whether drug resistance will develop in patients awaits further testing.

In the earlier phase I clinical trials when patients were treated with higher doses of dCF, prohibitive renal, neurologic, and ocular toxicities were noted.[20,25] We have recently reported that low doses of dCF (4 mg/m^2) administered to a large number of patients with chronic lymphocytic leukemia were well tolerated.[19] In both studies complications have been minimal with low-dose dCF. In fact, in our group of patients with hairy cell leukemia who were at high risk of infection due to neutropenia and monocytopenia, there was no apparent increase in infections observed.

This study was originally initiated because dCF had shown activity in heavily treated patients with chronic lymphocytic leukemia and other lymphoproliferative malignancies.[19,26] However, the exquisite sensitivity of hairy cell leukemia to the drug was unexpected. It has been suggested that the toxicity of dCF to normal as well as malignant lymphocytes can be explained by depletion of cellular nicotinamide-adenine dinucleotide (NAD) and subsequently adenosine triphosphate.[27] Evaluation of nucleotide pools and NAD in vivo following administration of dCF is currently being explored. The exact mechanism of tumor cell cytotoxicity, however, remains unknown

Since the description of hairy cell leukemia in 1958, the recommended management has been conservative with splenectomy being the treatment of choice in patients with progressive disease. Chemotherapy was infrequently used due to low response rates and significant toxicity. The success of dCF in our patients with progressive hairy cell leukemia including four who had not been splenectomized suggests that it also should be used in the nonsplenectomized patient regardless of spleen size. With the demonstration that two active agents exist for the treatment of hairy cell leukemia, the management of these patients needs reevaluation. Both alpha interferon and dCF offer significant palliation for this disease. Several multigroup studies are now underway to evaluate which of these agents is most appropriate for both the nonsplenectomized and splenectomized patients with progressive disease.

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REFERENCES

1. Bouroncle BA, Wiseman BK, Doan CA: Leukemic reticuloendotheliosis. Blood 13:609, 1958

2. Bouroncle BA. Leukemic reticuloendotheliosis, hairy cell leukemia Blood 53:412, 1979

3 Catovsky D: Hairy cell leukemia and prolymphocytic leukemia. Clin Hematol 6:245, 1977

4. Jansen J, Schuit HRE, Van Awit THL, Meizer LM, Hijmans W: Hairy cell leukemia, a B lymphocyte disorder. Br J Haematol 42:21, 1979

5. Jansen J, der Ottolander GJ, Schuit HR, Waayer JLM, Hijmans W: Hairy cell leukemia, its place among the chronic B cell leukemias. Semin Oncol 11:386, 1984

6. Jansen J, den Ottolander GJ, Holdrinet RSG, Tricot GJK, Hermans J: Prognosis and therapy in hairy cell leukemia. Semin Oncol 11:472, 1984

7. Golomb HM, Catovsky D, Golde DW: Hairy cell leukemia, a five-year update on seventy-one patients. Ann Int Med 99:485, 1983

8 Jansen J, Hermans J: Splenectomy in hairy cell leukemia. Cancer 47:2066, 1981

9. Mintz V, Golomb HM: Splenectomy as initial therapy in twenty-six patients with leukemic reticuloendotheliosis. Cancer Res 39:2366, 1979

10. Golomb HM, Mintz V: Treatment of hairy cell leukemia II, chlorambucil therapy in post splenectomy patients with progressive disease. Blood 54:305, 1979

11. Golomb HM. Progress reports on chlorumbucil therapy in post splenectomy patients with progressive hairy cell leukemia. Blood 57:464, 1981

12. Calvo F, Castachme S, Signaux F, Martoy M, Degos L, Bouron M, Flandrin G: Intensive chemotherapy of hairy cell leukemia in patients with aggressive disease. Blood 65:115, 1985

13. Stewart DJ, Benjamin RS, McCredie KB, Murphy S, Keating M: The effectiveness of rubidazone in hairy cell leukemia. Blood 54:298, 1979

14. Cheever MA, Fefer A, Greenberg PD, Applebaum F, Armitage JO, Buckner CD, Sale GE, Storb R, Witherspoon RP, Thomas ED: Treatment of hairy cell leukemia with chemoradiotherapy and identical twin bone marrow transplantation N Engl J Med 307:471, 1982

15. Ratain MJ, Golomb HM, Vardiman JW, Vokes EL, Jacobs RH, Daly K: Treatment of hairy cell leukemia with recombinant alpha 2 interferon. Blood 65:644, 1985

16. Quesada JP, Reuben J, Manning JT, Hersh EM, Gutterman JV: Induction of remission in hairy cell leukemia with alpha interferon. N Engl J Med 310:15, 1984

17. Jacobs AD, Champlin RE, Golde DW: Recombinant α-2 interferon for hairy cell leukemia. Blood 65:1017, 1985

18. Foon KA, Maluish AE, Abrams PG, Wrightington S, Stevenson HC, Alarif A, Fer MF, Overton WR, Poole M, Schnipper EF, Jaffe ES, Herberman RB: Recombinant leukocyte A interferon therapy for advanced hairy cell leukemia Am J Med 80:351, 1986

19. Grever MR, Leiby JM, Kraut EH, Wilson HW, Neidhart JA, Wall RL, Balcerzak SP: Low dose deoxycoformycin in lymphoid malignancy. J Clin Oncol 3:1196, 1985

20. Grever MR, Siaw MFE, Jacob WF, Neidhart JA, Miser JS, Coleman MS, Hutton JJ, Balcerzak SB: The biochemical and clinical consequences of 2'-deoxycoformycin in refractory lymphoproliferative malignancy. Blood 57:406, 1981

21. Spiers AS, Parekh SJ, Ramnes CR, Cassileth PA, Oken MM. Hairy cell leukemia: Pentostatin is effective both as initial treatment and after failure of splenectomy and alpha interferon. Am Soc Hem 66:718, 1985 (abstr)

22. Johnston JB, Glazer RI, Israels LG: 2'Deoxycoformycin, an effective treatment for hairy cell leukemia Am Soc Clin Onc 4:652-C, 1985 (abstr)

23. Johnston JB, Eisenhauer E, Barr R, Feldman L, Maksymiuk A, Scott G, Sutton D, Venner P, Walde D: 2'Deoxycoformycin (dCF) in hairy-cell leukemia (HCL). A Canadian Phase II Trial Proceedings of ASCO 5:159, 19896

24 Spiers ASD, De Conti RC, Parekh SJ, Ramnes CR, Oken MM, Eastern Cooperative Oncology Group. Hairy cell leukemia (HCL): Pentostatin (dCF, 2'Deoxycoformycin) as primary therapy, and after failure of splenectomy and alpha interferon (IFN). Proceedings of ASCO 5:151, 1986

25. Poplack DG, Sallan SE, Rivera G, Holcenberg J, Murphy SB, Blatt J, Lipton JM, Venner P, Glausiger DL, Ungerleider R, Johns D. Phase I study of deoxycoformycin in acute lymphoblastic leukemia. Cancer Res 41:3343, 1981

26. Grever MR, Wilson HE, Kraut EH, Neidhart JA, Balcerzak SP: Deoxycoformycin in the treatment of refractory chronic lymphocytic leukemia. Proc Am Soc Clin Oncol C-605, 1981 (abstr)

27 Seto S, Carrera CJ, Kubota M, Wasson DB, Carson DA. Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to non-dividing human lymphocytes J Clin Invest 75:377, 1985

Frequent and Extensive Deletion During the 9,22 Translocation in CML

By Daniel W. Popenoe, Kim Schaefer-Rego, J. Gregory Mears, Arthur Bank, and David Leibowitz

Chromosomal translocation is one mechanism by which cellular oncogenes may be activated during tumorigenesis. The translocation of the *abl* oncogene to the Philadelphia chromosome in chronic myelogenous leukemia (CML) results in a new RNA transcript that fuses sequence from chromosome 22 to sequence from the *abl* oncogene. This RNA presumably codes for a new *abl*-related protein product found in CML, the activity of which is different from the normal *abl* protein. The molecular structure of the translocation varies from patient to patient, and the individual variation in RNA transcript and protein product remains to be defined. This report describes the frequent occurrence of chromosomal deletion within the 9q+ chromosome during these translocations. The location of the deletions suggests that some mechanism maintains the chromosomal breakpoint on the Philadelphia chromosome within a limited region. These deletions complicate the interpretation of Southern blots as a means of detecting the translocation.

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THE Philadelphia (Ph) chromosome in chronic myelogenous leukemia (CML) was the first human chromosomal abnormality consistently associated with a malignancy.[1,2] The 9,22 translocation which creates the Ph chromosome moves the *abl* oncogene from chromosome 9 to chromosome 22. The extent of the normal cellular *abl* (*c-abl*) gene has not yet been defined, but the regions on chromosome 9 which are homologous to the transforming gene of the Abelson murine leukemia virus (*v-abl*) have been identified.[3,4] The breakpoint on chromosome 9 is sometimes found 4 to 6 kilobases (kb) 5′ to this *v-abl* homologous region, but is most often much further 5′.[5-7] The minimum amount of *abl* sequence from chromosome 9 which must move to chromosome 22 to generate a productive Ph chromosome is not yet known. In the cases thus far examined, the portions of *c-abl* that are homologous to the viral gene always translocate to the Ph chromosome. In contrast to the wide variation in the chromosome 9 breakpoint location, reports to date have suggested that the breakpoints on chromosome 22 occur within a region of approximately 6 kb, the breakpoint cluster region (bcr).[7]

The normal *abl* gene produces two RNA transcripts approximately 6 and 7 kb in size, and a protein with a molecular weight of 145 kilodaltons (kd).[8,9] After the *abl* gene is translocated to the Ph chromosome, a new 9 kb *abl*-related RNA species is produced.[10-12] This new RNA begins in the chromosome 22 sequence and continues across the translocation junction into the *abl* sequence.[13,14] This newly created fusion gene of chromosome 22 and chromosome 9-*abl* sequence presumably codes for a new protein found in CML with a molecular weight of 210 kd.[15-17] The protein kinase activity of this new protein has been reported to differ from that of the normal *abl* protein.[18]

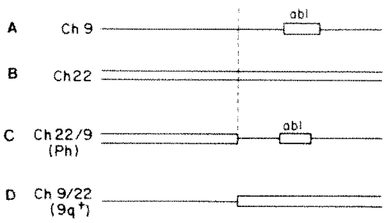

Fig 1. Schematic representation of the 9,22 translocation products and the production of the Ph chromosome. (A) Normal chromosome 9. The dashed line indicates the chromosomal breakpoint. The *v-abl* homologous region of the *abl* oncogene is indicated on chromosome 9. The breakpoint location on chromosome 9 is variable; in this example it is located 5′ to the region homologous to the *v-abl* gene. (B) Normal chromosome 22. The breakpoint location on chromosome 22 is within the bcr. (C) The Ph chromosome. The 5′ portion of the Ph chromosome comes from chromosome 22, the 3′ portion comes from chromosome 9. (D) Generation of the 9q+ chromosome. The 5′ portion of the 9q+ chromosome is from chromosome 9, the 3′ portion from chromosome 22.

The four chromosomes present before and after the translocation are diagrammed in Fig 1. It is possible to detect the breakage and reunion of the chromosomes by using DNA fragments from either chromosome 9 or chromosome 22 as probes to detect novel restriction fragments that span the translocation junction point.

The present study employed two probes from the bcr to localize the chromosome 22 breakpoints in CML patients. In this analysis, we demonstrate that deletion of chromosome 22 sequence occurs frequently during this translocation. However, even in the presence of these deletions, the 3′ end of the chromosome 22 portion of the Ph chromosome always terminates within the bcr, suggesting that there is some vital sequence located near the 5′ end of the bcr. In addition, the frequent occurrence of deletion in this area means that 3′ bcr probes will not identify the rearrangement in certain patients. To consistently identify the 9,22 translocation will at least require the use of a probe from the 5′ portion of the bcr.

MATERIALS AND METHODS

DNA was isolated from the peripheral white blood cells of CML patients using methods that have previously been described.[5] All the patients described in this study had a karyotype determined, and all patients had both Ph and 9q+ chromosomes. Some patients had additional chromosomal abnormalities. It was essential to obtain a

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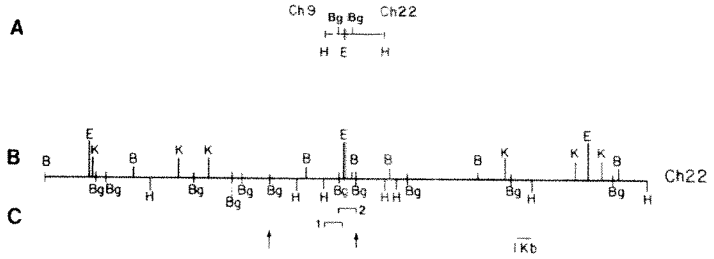

Fig 2. (A) The 9q+ chromosome junction clone from FP, a CML patient[5] is at the top. The 5′ end of the junction clone, shown in A, is sequence from chromosome 9, the 3′ end is chromosome 22 sequence. (B) A map of chromosome 22 derived from reference 7 and work in this laboratory. The Bgl II fragment of the clone in A was used to screen a normal human recombinant library to generate chromosome 22 clones and the fragments identified in C were used as probes. (C) Fragments of chromosome 22 used as probes. The Hind III/Eco RI fragment, #1, is referred to as the 5′ probe. The Bgl II fragment, #2, is the 3′ probe. The vertical arrows indicate the bcr as defined in Groffen et al.[7]

fragment of chromosome 22 to analyze the patients' breakpoints. Restriction mapping of the patient FP, described in an earlier publication,[5] had shown that the 9q+ chromosome translocation junction point was contained within a 5.0 kb Hind III fragment (Fig 2). A recombinant DNA library was constructed in the phage vector charon 21a, and approximately 1 × 10^6 phage were screened using a Hind III-Eco RI fragment of chromosome 9 located just 5′ of the *v-abl* homologous region.[5] The 5 kb Hind III fragment containing the translocation junction was isolated and subcloned into the bacteriophage pSP64 (Promega Biotech, Madison, Wis). The Bgl II fragment of this clone (probe 2 in Fig 2) was then used to screen a normal human recombinant library. A clone containing the normal 4.2 kb Hind III fragment of chromosome 22 was isolated, and the 1.2 kb Hind III-Eco RI fragment (labelled probe 1 in Fig 2) was subcloned from it. These two probes were used to examine Southern blots of CML patients. These blots generated the data shown in Figs 4 and 5, and Tables 1 and 2.

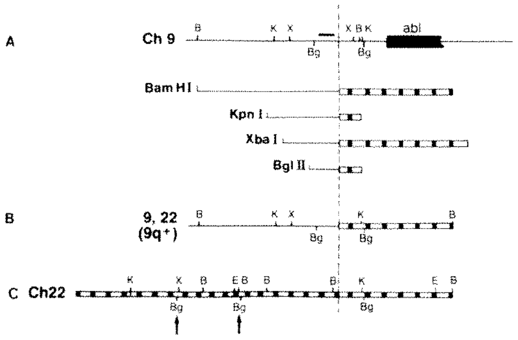

Fig 3. Restriction analysis of the 9q+ chromosome from patient TH (from reference 5). (A) The top line is a map of chromosome 9, the region labelled *abl* indicates the 5′ end of the *v-abl* homologous region; the solid black bar above the restriction map indicates a fragment of chromosome 9 used as a probe on Southern blots. The lines labelled Bam HI, Kpn I, Xba I, and Bgl II depict the abnormal restriction fragments identified on Southern blots by the chromosome 9 probe when DNA from patient TH is digested with the respective enzymes. The sizes of the abnormal fragments are Bam HI, 24 kb; Kpn I, 5.4 kb; Xba I, 15 kb; Bgl II, 4.4 kb.[5] These new restriction fragments are from the 9q+ chromosome. The thin black line indicates the portion of the fragment that is from chromosome 9, the hatched portion of the line represents the fragment derived from chromosome 22. (B) Restriction map of the 9q+ chromosome based upon the restriction data from A. The chromosome 9 probe localizes the breakpoint on chromosome 9. The size of the abnormal restriction fragments is known from Southern blots. The known restriction map of normal chromosome 22 in C, together with these restriction data make it possible to generate the restriction map of the chromosome 22 portion of the 9q+ chromosome. (C) The vertical dashed line indicates the chromosomal breakpoint. The alignment of the 9q+ chromosome with the normal chromosome 22 map indicates that the portion of chromosome 22 that became part of the 9q+ chromosome terminated roughly 8 to 10 kb 3′ of the bcr (shown by the vertical arrows below C at Bgl II sites). This chromosome 22 breakpoint which joins the 9q+ chromosome will be referred to as the 9q+-22-bkpt. The letters indicate restriction sites for the enzymes Bam HI, Kpn I, Xba I, Bgl II, and Eco RI.

RESULTS

Using a *v-abl* fragment as a probe to screen a normal human recombinant library permitted the isolation of human

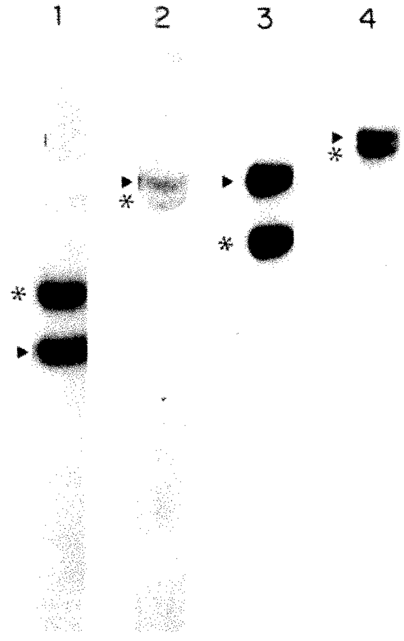

Fig 4. Southern blots of 2 patients and 1 CML cell line probed with the more 3′ bcr probe, probe 2. In each sample, a normal band is seen, indicated by an arrowhead, plus an additional abnormal band indicated by an asterisk. This abnormal band represents a translocation junction fragment (Figs 1 and 2). Lane 1: patient MK, enzyme Hind III, normal 4.2 kb band, abnormal 6.5 kb band. Lane 2: cell line EM2, Xba I, normal 9.4 kb band, abnormal 8.0 kb band. Lane 3: patient FP, Xba I, normal 9.4 kb band, abnormal 5.4 kb band. Lane 4: patient FP, Kpn I, normal 20 kb band, abnormal 18 kb band.

c-abl fragments which were then employed as probes to examine Southern blots of CML patients.[5] These probes identified two patients whose chromosome 9 breakpoints were within 4 to 6 kb of the *v-abl* homologous region.[5] The restriction analysis of the 9q+ chromosome from one of these patients, TH, is summarized in Fig 3. The restriction map of the 9q+ chromosome from TH provides a linear map of the restriction enzyme sites located in chromosome 22 sequence immediately 3′ to the 9,22 junction. By aligning these restriction sites with the known restriction map of chromosome 22 (Fig 3), these data suggest that the chromosome 22 breakpoint in the 9q+ chromosome is not within the bcr in patient TH.

To further localize the chromosome 22 breakpoint in TH, and to identify the chromosome 22 breakpoints in other CML patients, cloned probes from chromosome 22 were used. The probes labelled 1 and 2 in Fig 2 were used to examine Southern blots of 14 CML patients and 1 CML cell line (Table 1). Representative lanes from these Southern blots are shown in Figs 4 and 5. The more 3′ chromosome 22 probe, #2 in Figure 2C, identified breakpoints within the bcr in 6 of our 14 patients. The abnormal restriction bands found in the two CML patients and the CML cell line shown in Fig 4 indicate that a breakpoint within the bcr is present. This is consistent with previously reported data.

The individual whose Southern blots are shown in Fig 5A is the patient TH, whose 9q+ chromosome digests were summarized in Fig 3, and who represents a group of 4 patients who were initially perplexing. All these patients had Ph and 9q+ chromosomes visualized in a karyotype. The chromosome 22 probe labelled #1 in Fig 2 identified not only the normal but also an additional abnormal restriction band (Fig 5A, lanes 3 to 5). These bands are drawn schematically in Fig 6. This indicated that the patient had a fragment of chromosome 22 that ended within the bcr joined to a fragment of chromosome 9. Figure 5A, lane 3, shows the abnormal 7.1 kb Bgl II band; lane 4, the abnormal 13 kb Kpn I band; and lane 5, the abnormal 4.4 kb Bam HI band which are shown in Fig 6 aligned with the map of chromosome 22. These abnormal restriction bands make it possible to define the end of the chromosome 22 fragment that becomes part of the Ph chromosome. As shown in Fig 6, chromosome 22 sequence on the Ph chromosome ends within the bcr for patient TH. We will call the end of chromosome 22 which joins the Ph chromosome the Ph chromosome/chromosome 22 breakpoint (Ph-22-bkpt). This data from chromosome 22 probes contradicted the earlier data summarized in Fig 2 that indicated that the chromosome 22 breakpoint on the 9q+ chromosome was located at least 8 kb 3′ to the bcr. The simplest explanation for these data is that the chromosome 22 sequence lying between the Ph-22-bkpt and the 9q+ chromosome/chromosome 22 breakpoint (9q+-22-bkpt) had been deleted during the translocation. This is illustrated in Fig 7.

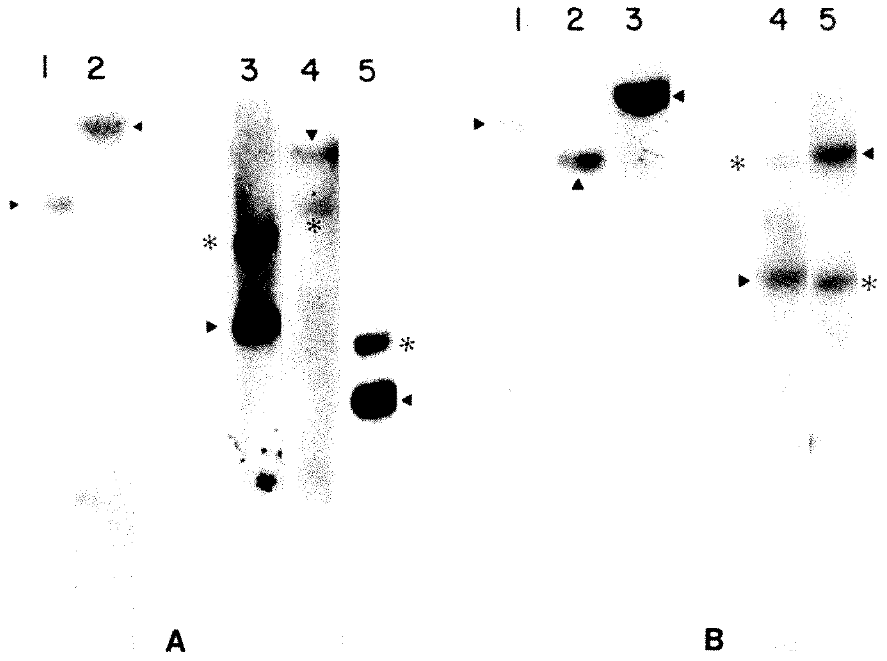

Fig 5. (A) Southern blots of a single patient, TH, representing the group of patients who showed only normal bands with the more 3′ chromosome 22 probe, but were found to have breakpoints within the bcr when probed with the more 5′ chromosome 22 probe. Arrowhead indicates normal band; asterisk indicates abnormal band. The lanes are from different gels, so fragment size and distance migrated are not comparable from one lane to another. Lanes 1–2: Probe 2, the 3′ probe. Lanes 3–5: Probe 1, the 5′ probe. Lane 1: Xba I digest with normal 9.4 kb band. Lane 2: Eco RI digest with a normal 16.7 kb band. Lane 3: Bgl II digest, normal 4.9 kb band, abnormal 7.1 kb band. Lane 4: Kpn I digest, normal 20 kb band, abnormal 13 kb band. Lane 5: Bam HI digest, normal 3.2 kb band, abnormal 4.4 kb band. The 3 abnormal bands are illustrated in Fig 6. (B) Southern blots of a second patient, ES, who also had a bcr breakpoint when probed with probe 1, but showed only normal bands when probed with probe 2. Lane 1: probe 2, Eco RI, normal 16.7 kb band. Lane 2: probe 2, Xba I, normal 9.4 kb band. Lane 3: probe 2, Kpn I, normal 20 kb band. Lane 4: probe 1, Bgl II, normal 4.9 kb band, abnormal 8.0 kb band. Lane 5: probe 1, Eco RI, normal 17.1 kb band, abnormal 7 kb band.

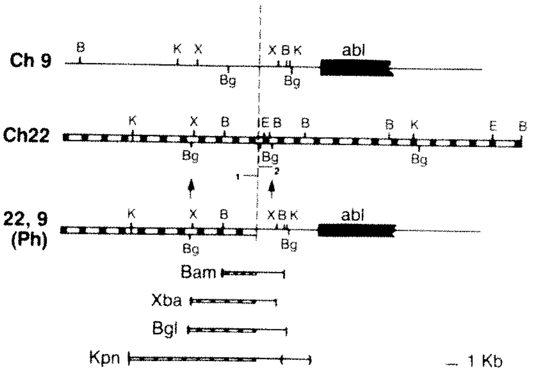

Fig 6. Ph chromosome translocation for the patient TH. Line 1: Map of normal chromosome 9. Solid black region represents the 5′ end of the region homologous to *v-abl*. Vertical dashed line represents location of chromosome break. Line 2: Map of normal chromosome 22. Vertical arrows below the line indicate the extent of the bcr. Horizontal lines labelled 1 and 2 represent chromosome 22 fragments used as probes. Line 3: Map of the Ph chromosome for patient TH. Hatched region is sequence from chromosome 22, solid line is from chromosome 9. The location of the chromosome 22 breakage which joined the Ph chromosome (Ph-22-bkpt) was determined by the appearance of abnormal restriction bands in Southern blots of DNA from patient TH. The probe for these blots was probe 1, and the abnormal bands are indicated below Line 3. The restriction enzyme that generated each new band is shown to the left of the band. The 5′ portion of each fragment is from chromosome 22, as is indicated by the hatched line; the 3′ portion is from chromosome 9, as indicated by the solid line. Three of these abnormal bands are seen in the blots in Fig 5A: Lane 5, Bam HI, 4.4 kb abnormal fragment; Lane 3, Bgl II, 7.1 kb abnormal fragment; Lane 4, Kpn I, 13 kb abnormal fragment. The length of the Kpn fragment has varied slightly on different Southern blots.

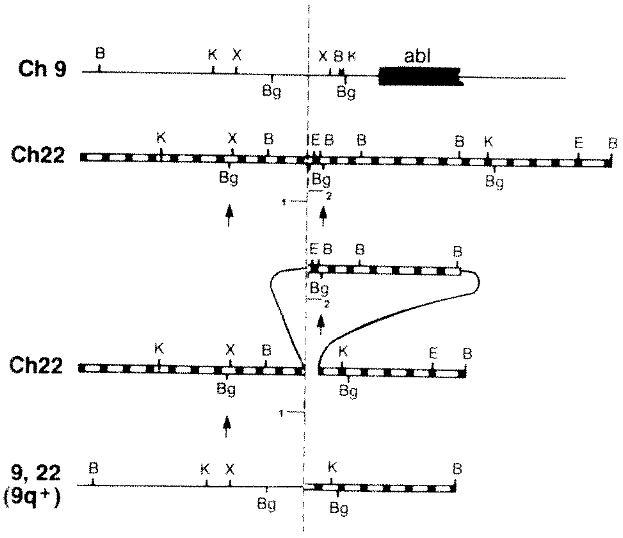

Fig 7. Diagram of deletion during the 9,22 translocation in patient TH. Lines 1 and 2 are as seen in Fig 6. The vertical dashed line indicates the point of chromosome breakage for the creation of the Ph chromosome, as shown in Fig 6. Line 4 is the restriction map for the 9q+ chromosome in TH, as illustrated in Fig 3. Line 3: Illustrates the region of chromosome 22 that was lost during this translocation. The deleted region includes that represented by probe 2. Since probe 2 sequence is not present in the 9q+ chromosome, Southern blots with probe 2 will show only normal restriction fragments, as shown for patient TH in Fig 5A, lanes 1–2, and for a second similar patient in 5B.

This conclusion was supported by the finding that probe 2 in Fig 2 identified only normal bands on Southern blots of the patient TH. Since the data summarized in Fig 6 indicated that the Ph-22-bkpt in patient TH was adjacent to probe 1, it would have been expected that in the absence of deletion probe 2 would identify the 9q+ chromosome fragments. In fact, probe 2 identified only normal restriction bands on Southern blots of the patient TH (Fig 5A, lanes 1 and 2). This implies that probe 2 sequence was not present in the 9q+ or Ph chromosome. The restriction data on the 9q+ chromosome generated by the chromosome 9 probe indicate that the 9q+-22-bkpt is located roughly 8 kb 3′ to the bcr. As shown in Fig 7, taken together, these data indicate that a region of chromosome 22 extending from probe 1 to the 9q+-22-bkpt is lost during the translocation. This deleted fragment is 8 to 10 kb.

Similar results are shown for another patient in Fig 5B. The more 5′ bcr probe clearly identifies a breakpoint within the bcr, whereas the 3′ probe detects only normal restriction fragments. This again implies that a portion of chromosome 22 located on the 3′ side of the Ph-22-bkpt was deleted during the translocation. This pattern was seen in two additional patients.

There is an additional group of four patients who show only normal restriction fragments with both chromosome 22 probes. When probes from further 5′ on chromosome 22 are available it will be possible to determine whether these patients also have a Ph-22-bkpt with a 3′ deletion. Though not previously observed, it is also possible that these patients have breakpoints outside the bcr. The results for the entire group of patients are presented in Table 1 and summarized in Table 2.

DISCUSSION

We have documented variable deletions occurring in the region of chromosome 22 that becomes part of the 9q+ chromosome. These data are consistent with the hypothesis that the physiologically significant chromosome in CML is the Ph chromosome, and that it is not crucial to conserve the breakpoint region that becomes part of the 9q+ chromosome in the 3′ portion of chromosome 22. In contrast, since the new *abl* transcript and protein originate from the Ph chromosome, it appears vital to connect the portion of chromosome 9 carrying the *abl* locus to chromosome 22 at a breakpoint that preserves a specific chromosome 22 region extending 5′ from within the bcr. Our results also indicate that most of the patients have a 5′ chromosome 22 break that lies within the bcr despite deletions of more 3′ chromosome 22 sequence. We have observed only a few CML patients whose Ph-22-bkpts may be outside the bcr. It will be important to determine the precise breakpoint in these individuals, and whether they generate abnormal *abl* RNA transcripts and proteins.

There are several possible explanations for the consistent generation of the Ph chromosome and its translocation in

Table 1. Probe 1 (5′ Probe) and Probe 2 (3′ Probe)

	Probe 1				
	Bgl II	Kpn I	Bam HI	Eco RI	Xba I
Abnormal fragments identified with the 3′ bcr probe					
MK (see data for probe 2)					
EM2 (see data for probe 2)					
FP (see data for probe 2)					
MP (see data for probe 2)					
GP (see data for probe 2)					
DA (see data for probe 2)					
Normal fragments with the 3′ probe/abnormal with the 5′ probe					
TH	4.9/7 1*	20/13*	3.2/4 4*		
ES	4.9/8.0*			17.1/7*	
Co	4 9		3.2/7*	17.1/27*	
Bn	4.9/8.4*				
Normal fragments with 3′ and 5′ probes					
St	4 9		3.2		
HO	4 9				
CD	4.9			17.1	9.4
Li	4.9			17.1	9.4

	Probe 2				
	Hind III	Xba I	Kpn I	Eco RI	Bam HI
Abnormal fragments identified with the 3′ bcr probe					
MK	4.2/6 5*	9.4/3.8*			3.2/2 5/19*
EM2		9.4/8 0*			
FP		9.4/5.4*	20/18*		
MP	4 2/3.6*				3.2/2 5/1.9*
GP			20/18*		3 2/2 5/2.8*
DA		9.4/12.2*	20/12.5*		
Normal fragments with the 3′ probe/abnormal with the 5′ probe					
TH	4.2	9.4		16 7	
ES		9.4	20	16.7	3.2/2.5
Co					3 2/2.5
Bn	4 2	9.4	20		3.2/2.5
Normal fragments with the 3′ and 5′ probe					
St	4.2				3 2/2.5
HO		9.4	20	16.7	
CD		9.4	20	16 7	3.2/2 5
Li				16.7	

Numbers indicate size in kb for the fragments seen on Southern blots with the indicated enzymes and probes These patients are summarized in Table 2.

*The band is abnormal.

CML. It is possible that the 9,22 translocation is one of many chromosomal translocations that occur at random in human cells, or is one that is favored by the environment of early hematopoietic precursors. The environment established by previous changes within the abnormal hematopoietic stem cell might provide a selective advantage so that a clone of cells with this translocation enlarges and results in the leukemia. There may be earlier events, and also perhaps subsequent events, in addition to the formation of the Ph chromosome, that are required for the appearance of the phenotype that we recognize as CML.[19,20] The question is to determine how these other events relate to the 9,22 translocation. Our data suggesting that translocation of the *abl* locus to a point within the bcr is crucial for the appearance of the CML phenotype emphasizes the potential etiologic role of this event. A role of the bcr in this translocation is also supported by observations that a DNAase I hypersensitive site is present 5′ to the bcr in the CML-derived cell line K562 (Mears JG, submitted). Taken together, the results support the hypothesis that in the formation of the Ph chromosome *abl* must to be translocated to a specific transcriptionally active region of chromosome 22. Further investigation of the precise molecular changes occurring during this transloca-

Table 2. Restriction Fragments Seen in Southern Blots of CML Patients Probed With Chromosome 22 Probes

Patients with abnormal fragments identified with the 3′ bcr probe	6
Normal fragments with 3′ probe/abnormal with 5′ probe	4
Normal fragments with 3′ and 5′ probes	4*

*Some of these patients may have 5′ breakpoints within the bcr that will become apparent with more 5′ chromosome 22 probes.

tion should provide additional insight into the involvement of the *abl* gene in the generation of this leukemia.

We have shown that deletion is a frequent occurrence during the 9,22 translocation that creates the Ph chromosome in CML. Despite the loss of as much as 8 to 10 kb of chromosome 22 sequence in the case that is best characterized, some mechanism has operated to maintain the Ph-22-bkpt within the bcr. To date, karyotype analysis has been the method used to detect the 9,22 translocation. There are situations in which this method is inadequate, as when the patient sample does not provide interpretable metaphases. There are also three reported cases in which a 9,22 translocation was documented by Southern blotting even in the absence of an identifiable Ph chromosome.[21-23] In these situations, it would be advantageous to use Southern blotting rather than karyotype analysis to identify the translocation. The frequent occurrence of deletion during this translocation complicates the interpretation of such restriction analysis. From our data, a probe from the 3′ portion of the bcr is inadequate in a significant number of patients. The most desirable probe for detecting the rearrangement would be a chromosome 22 fragment located 5′ of all Ph chromosome breakpoints. If all patients have a chromosome 22 breakpoint within the 5.8 kb bcr, then a fragment from the 5′ end of the bcr would be an acceptable probe. More data on the location of chromosome 22 breakpoints and the extent of deletion in patients with a Ph chromosome will be required to determine what probe will be most reliable for reproducibly detecting the 9,22 translocation.

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REFERENCES

1 Nowell PC, Hungerford DA: A minute chromosome in human CML. Science 132:1498, 1960

2 Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and giemsa staining. Nature 243.290, 1973

3. Heisterkamp N, Groffen J, Stephenson JR, Spurr NK, Goodfellow PN, Solomon E, Carritt B, Bodmer WF: Chromosomal localization of human cellular homologues of two viral oncogenes. Nature 299.747, 1982

4. Heisterkamp N, Groffen J, Stephenson JR. The human *v-abl* cellular homologue. J Mol Appl Genet 2:57, 1983

5. Leibowitz D, Schaefer-Rego K, Popenoe DW, Mears JG, Bank A: Variable breakpoints on the Philadelphia chromosome in chronic myelogenous leukemia. Blood 66 243, 1985

6 Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR, Grosveld G: Localization of the *c-abl* oncogene adjacent to a translocation breakpoint in CML. Nature 306 239, 1983

7. Groffen J, Stephenson JR, Heisterkamp N, deKlein A, Bartram CR, Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 36 93, 1984

8. Westin EH, Wong-Staal F, Gelmann EP, Dalla-Favera R, Papas TS, Lautenberger JA, Eva A, Reddy EP, Tronick SR, Aaronson SA, Gallo RC: Expression of cellular homologues of rv *onc* genes in human hematopoietic cells. Proc Natl Acad Sci 79:2490, 1982

9. Witte ON, Rosenberg N, Baltimore D: A normal cell protein cross-reactive to the major Abelson murine leukemia virus gene product. Nature 281:396, 1979

10. Leibowitz D, Cubbon RM, Bank A. Increased expression of a novel *c-abl* related RNA in K562 cells. Blood 65.526, 1985

11. Gale RP, Canaani E: An 8 kilobase *abl* RNA transcript in chronic myelogenous leukemia. Proc Natl Acad Sci 81.5648, 1984

12. Collins SJ, Kubonishi I, Miyoshi I, Groudine MT: Altered transcription of the *c-abl* oncogene in K562 and other chronic myelogenous leukemia cells. Science 225:72, 1984

13. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of *abl* and bcr genes on chronic myelogenous leukaemia Nature 315.550, 1985

14 Heisterkamp N, Stam K, Groffen J, de Klein A, Grosveld G: Structural organization of the bcr gene and its role in the Ph translocation. Nature 315:758, 1985

15. Konopka JB, Watanabe SM, Witte ON. An alteration of the human *c-abl* protein in K562 leukemia cells unmasking associated tyrosine kinase activity. Cell 37:1035, 1984

16. Konopka JB, Watanabe SM, Singer JW, Collins SJ, Witte ON: Cell lines and clinical isolates derived from Ph-positive chronic myelogenous leukemia patients express *c-abl* proteins with a common structural alteration. Proc Natl Acad Sci 82:1810, 1985

17. Kozbor D, Giallongo A, Sierzega ME, Konopka JB, Witte ON, Showe LC, Croce CM. Expression of a translocated *c-abl* gene in hybrids of mouse fibroblasts and chronic myelogenous leukemia cells. Nature 319:331, 1986

18. Konopka JB, Witte ON. Detection of *c-abl* tyrosine kinase activity in vitro permits direct comparison of normal and altered *abl* gene products. Mol Cell Biol 5:3116, 1985

19. Fialkow PJ, Martin PJ, Najfeld V, Penfold GK, Jacobson RJ, Hausen JA. Evidence for a multistep pathogenesis of chronic myelogenous leukemia. Blood 58.158, 1981

20. Lisker R, Caras L, Mutchinich O, Perez-Chavez F, Labardini J: Late appearing Philadelphia chromosome on two patients with chronic myelogenous leukemia. Blood 56.812, 1980

21. Bartram CR, Kleihauer E, deKlein A, Grosveld G, Teyssier JR, Heisterkamp N, Groffen J: *C-abl* and bcr are rearranged in a Ph negative CML patient. EMBO J 4:683, 1985

22. Stam K, Heisterkamp N, Grosveld G, deKlein A, Verma RS, Coleman M, Dosik H, Groffen J: Evidence of a new chimeric bcr/*c-abl* mRNA in patients with chronic myelogenous leukemia and the Philadelphia chromosome. N Engl J Med 313:1429, 1985

23. Bartram CR, Carbonell F: bcr rearrangement in Ph-negative CML. Cancer Genet Cytogenet 21:183, 1986

Growth and Development Following Marrow Transplantation for Leukemia

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One hundred forty-two patients between the ages of 1 and 17 years who survived disease-free more than 1 year after marrow transplantation for hematologic malignancy had growth and development evaluations from one to 14 years posttransplant (median 4 years). Prior to transplant all children received multiagent chemotherapy and 55 also received central nervous system irradiation, but none had growth and development evaluations. Marrow transplant preparation included high-dose chemotherapy and total body irradiation (TBI) given as a single dose of 9.2 to 10.0 Gy (79 patients) or as fractionated doses of 2.0 to 2.25 Gy/d for six to seven days (63 patients). After transplant abnormal thyroid function was present in 39%. Stimulated 11-desoxycortisol levels were subnormal in 24% of patients evaluated. Growth hormone (GH) deficiency was present in 17 of 25 children who received previous cranial irradiation. Partial GH deficiency was present in 4 of 25 who received previous cranial irradiation and in 6 of 18 who had not received cranial irradiation. Height velocity was decreased in all patients. After transplant, height was significantly influenced by chronic graft-v-host disease and single-dose TBI. Sixty-eight percent had delayed development of secondary sexual characteristics. Gonadal failure occurred in nearly all who were postpubertal at transplant. While it is not possible to determine how many of these endocrine abnormalities occurred as a result of treatment administered prior to transplantation, these data do demonstrate that children who become long-term survivors after marrow transplantation for hematologic malignancy have endocrine abnormalities that adversely affect growth and development.

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OVER the past 15 years the success of marrow transplantation for the treatment of children with malignant hematologic disorders has increased steadily. The number of patients transplanted and the number of long-term survivors have increased. Transplant preparative regimens include high-dose cyclophosphamide (CY) plus total body irradiation (TBI).[1-8] Among young adults the doses of irradiation used in the transplant preparative regimen affect thyroid and gonadal function.[9-11] Cranial irradiation administered prior to marrow transplantation adversely influences growth hormone production.[12-17] Glucocorticoid treatment, often given for chronic graft-*v*-host disease (GVHD), also adversely influences growth.[18,19] The development of endocrine abnormalities in children following TBI and marrow transplantation is of importance because of the possible influence on normal growth and development. Therefore, we evaluated endocrine function and long-term effects on growth and development in 142 children transplanted for hematologic malignancies following preparation with CY and TBI.

MATERIALS AND METHODS

Patients

We reviewed the records of 155 consecutive children given marrow transplants in Seattle between 1970 and April 1984 who survived in remission more than 1 year after transplantation. Treatment prior to referral for transplantation varied with referring institution and time of original diagnosis. Since height and developmental information from diagnosis and time points prior to referral was infrequently available, the data included in this report begin at the time of transplant. Analyses included only patients less than 18 years of age at the time of transplant Posttransplant information was obtained on 142 of the 155 patients, who form the basis of this report. These patients included 52 girls and 90 boys with a median age of 10 years (range 1 to 17) at the time of transplantation. The time of evaluation was 1 to 14 (median 4) years after transplant Fifty-nine patients had acute lymphoblastic leukemia, 55 had acute nonlymphoblastic leukemia, 15 had Ph positive chronic myelogenous leukemia, 5 had juvenile chronic myelogenous leukemia, 4 had preleukemia, and 4 had lymphoma. Nine patients received grafts from syngeneic donors, 111 from HLA-identical sibling donors, 21 from partially HLA-identical family members, and 1 received autologous marrow. Prior to referral for transplantation all patients had received multiagent chemotherapy, 55 patients had received a median exposure of 24.0 (range 18.0 to 29.0) Gy of cranial irradiation, 5 of these 55 had also received a median exposure of 12.0 (range 8.0 to 24.0) Gy of spinal irradiation and 9 boys had received a median exposure of 20.0 (range 18.0 to 24.0) Gy of testicular irradiation.

Conditioning, Marrow Transplantation, and Follow-up Evaluations

In preparation for marrow transplant, all patients received cyclophosphamide, 60 mg/kg on each of two consecutive days followed by TBI.[20,21] TBI was delivered from dual opposing ^{60}Co sources at a dose rate of 5.5 to 8 cGy/min measured in air at the midpoint. Seventy-nine patients received 9.2 to 10.0 Gy in a single exposure and 63 patients received a fractionated regimen, of 2 0 to 2 25 Gy/d for six to seven days for cumulative doses of 12.0 to 15.75 Gy.[22] Marrow was infused within four hours of completing TBI (day 0). Details of the marrow grafting procedure and management of the patients after grafting have been described.[19-28]

Height, weight, and physical examinations were performed on all patients at yearly intervals following transplant. Tanner and Whitehouse pubertal developmental standards for breast, pubic hair, and

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genital maturity were used to evaluate secondary sex characteristic development among girls and boys who were prepubertal at the time of transplant and were now more than 12 and 13 years of age, respectively.[29,30] Other parameters tested on many included bone age, serum thyroid stimulating hormone (TSH), thyroxine (T4), follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and estradiol. In patients not receiving glucocorticoids, 11-desoxycortisol levels after metapyrone stimulation and growth hormone (GH) levels following L-dopa or insulin stimulation were measured. Bone age was determined according to the standards of Greulich and Pyle.[31] All values were related to the patient's age at the time studies were performed. Height age and growth velocity were calculated for each patient from standard stature curves.[32] Mean height velocity values were plotted on standard velocity curves.[29,30] Change in height age was determined using the ratio of year of height age to year of chronologic age. All protocols and consent forms were approved by the Human Subjects Review Committee of Fred Hutchinson Cancer Research Center.

Statistical Evaluation

Univariate comparisons used the Mantel-Haenszel method for multidimensional contingency tables.[33] Multivariate analysis used a proportional hazards regression model and included the covariates of previous cranial irradiation, TBI regimen, chronic GVHD, sex, and age.[34,35] The analysis of variance test was used to evaluate height and age variables. The assumption of homogeneous variances was tested and met.[36] Comparability between treatment groups for height and age at the time of transplant was established by examining height, age, sex, and history of prior cranial irradiation. Subsequent analyses examined height and changes in height with respect to the impact of cranial irradiation, TBI regimen, and chronic GVHD.

RESULTS

Thyroid Function

Thyroid hormone levels were evaluated in 116 asymptomatic patients on 181 occasions from 1 to 11 years following transplant. A total of 45 patients (39%) had abnormal TSH and/or T_4 levels. Decreased thyroid function reserve with elevated TSH levels (>6 3 mU/L) and normal T_4 levels (7.3 to 15 μg/dL for 1 to 5 years, 6.4 to 13 μg/dL for 6 to 10 years, 5.6 to 12 μg/dL for 11 to 15 years, 4.5 to 12.5 μg/mdL for ≥16 years) was demonstrated in 18 patients. Thirteen patients had normal or elevated TSH levels and low T_4 levels. Six of these 31 receive thyroid hormone supplement. Seven patients had normal TSH and elevated T_4 values and 7 had both elevated TSH and T_4 values. One of these developed a thyroid adenoma which was discovered at autopsy 9.5 years after transplant.

The age at transplant for the 45 patients with abnormal thyroid function ranged from 2 to 16 (median 8) years. Among the evaluated patients, 53% were tested once, 34% were tested twice, and 13% were tested on three occasions. The percent of patients developing abnormalities each year remained relatively constant with increasing time after transplant. Multivariate analyses demonstrated no significant correlation of any abnormal thyroid function with previous cranial irradiation, single exposure or fractionated TBI, presence or absence of chronic GVHD, or patient age or sex.

Adrenocortical Function

Plasma 11-desoxycortisol (compound S) levels were measured after metyrapone stimulation on 87 occasions in 78 patients who were 1 to 8 years after transplant and not receiving glucocorticoid treatment. Nineteen patients (24%) had subnormal 11-desoxycortisol levels (stimulated level <5). None of the patients were symptomatic, all had normal electrolytes, and none received treatment.

The patients with subnormal levels were from 2 to 15 years of age (median 6 years) and the percent developing abnormalities remained relatively constant with increasing time after transplant. Univariate and multivariate analyses did not demonstrate a correlation with age, sex, previous cranial irradiation, TBI regimen, or chronic GVHD.

Growth Hormone

GH levels were determined following L-dopa or insulin stimulation on 52 occasions in 43 patients not receiving glucocorticoid treatment from 1 to 8 years (median 3) following transplant. GH deficiency (values <7 ng/dL) was present in 17 of 25 evaluated patients who had received pretransplant cranial irradiation. These 17 children were from 2 to 13 (median 8) years of age at transplant and their ages at initial cranial irradiation ranged from 1 to 12 (median 5) years. Partial GH deficiency (values >7 to 10 ng/dL) was present in 10 of 26 children. Four of these 10 had received previous cranial irradiation.

Growth

Height Figure 1 illustrates height velocity curves. Substantial differences were noted between patients with and without chronic GVHD. Among those with chronic GVHD, growth velocity of 5 patients who did not receive glucocorticoid treatment was not distinguishably different from the growth velocity of those who were treated. Catch-up growth was not observed after treatment for chronic GVHD was stopped. The change in the patient's height age, expressed as a ratio of year of height age to chronological age, continued to decrease with increasing number of years posttransplant (Fig 2).

Analyses of variance tests were used to evaluate the influence of chronic GVHD, type of TBI received, and previous cranial irradiation on linear growth. The time periods of ≤2 years and ≥3 years after transplant were used to evaluate growth with and without the influence of active chronic GVHD and/or its treatment. During the first 2 years after transplant boys without chronic GVHD grew significantly more (4.7 ± 0.32 standard error centimeters [cm]/yr) than boys with chronic GVHD (3.4 ± 0.32 cm/yr; $P < 0.0001$). By 3 or more years after transplant, boys who received single exposure TBI were 8.0 ± 2.3 cm shorter than boys who received fractionated exposure TBI ($P < 0.03$). Cranial irradiation had a significant influence in boys only when considered with type of TBI or with chronic GVHD. Among boys who had not received cranial irradiation, those given fractionated TBI were 15.2 ± 3.2 cm taller than those given single exposure TBI ($P < 0.04$). Cranial irradiation evaluated with chronic GVHD showed that boys who had

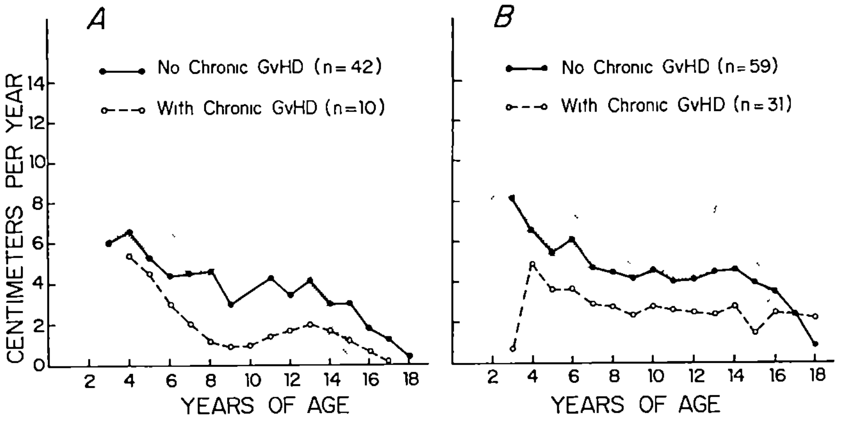

Fig 1. Growth velocity in centimeters growth per year of chronologic age for 52 girls (A) and 90 boys (B) following transplantation. Solid line illustrates growth velocity for 42 girls and 59 boys without chronic GVHD. Solid circles represent the mean centimeters growth for 2 to 13 (median 9) girls and 3 to 19 (median 10) boys at each age. Dashed line illustrates the growth velocity for 10 girls (A) and 31 boys (B) with chronic GVHD. Open circles represent the mean centimeters growth for 1 to 3 girls and 1 to 9 (median 6) boys at each age. Shaded area is the 10th to 90th longitudinal whole-year percentiles and is adapted from charts prepared by Tanner and Whitehouse.[29,30]

previous cranial irradiation and chronic GVHD were 21.7 cm ± 3.3 cm shorter than those who did not develop chronic GVHD ($P < 0.02$). Girls showed the same trends, but none of the differences were significant.

Bone age. Bone age, determined for 83 patients on 112 occasions from 1 to 9 years after transplant, was within 2 years of the chronologic age and height age for 73 patients, 18 of whom had chronic GVHD. For 10 patients, bone age lagged behind chronologic age more than 2 years but was appropriate for height age. Among these 10, 5 had chronic GVHD treated with glucocorticoids and one had chronic GVHD that was not treated.

Pubertal Development

Girls. Thirty-five girls ages 2 to 12 (median 8) years were prepubertal at transplant. Currently, 16 of the 35 are between 12 and 18 years of age (median 14) and are progressing through various stages of pubertal development. Figure 3 illustrates their developmental variation from normal. Six girls achieved menarche 2.3 to 5.5 years after transplant when they were between 12.7 and 14.5 years of age. Four of these 6 had received fractionated TBI. Ten girls have delayed development of secondary sexual characteristics.

Gonadotropin and estradiol levels were determined for 11 of the 16 girls over the age of 12 years. Four have normal LH, FSH, and estradiol levels. Two of these 4 initially had elevated LH and FSH determinations which returned to normal at 4 to 5 years after transplant. Elevated LH and FSH levels, >33 mIU/mL, were noted in 7 girls who also had estradiol levels <20 pg/dL and delayed onset of pubertal development.

Boys. Sixty-three boys between the ages of 1 and 13 (median 8) years were prepubertal at transplant. Thirty-one of these boys are currently between 13 and 22 years (median 16). Figure 4 illustrates their developmental variation from normal. Twenty-one have delayed development of secondary sexual characteristics.

Gonadotropin and testosterone levels were determined for 25 of the 31 developing boys. Three boys with prepubertal levels of LH, FSH, and testosterone obtained between 13 and 14 years of age all had delayed development. Five of 12 with normal LH also had normal FSH levels, testosterone levels, and developmental scores. Seven of 12 with normal LH levels had elevated FSH values and testosterone values were >300 ng/dL in 5 who had normal developmental scores and were

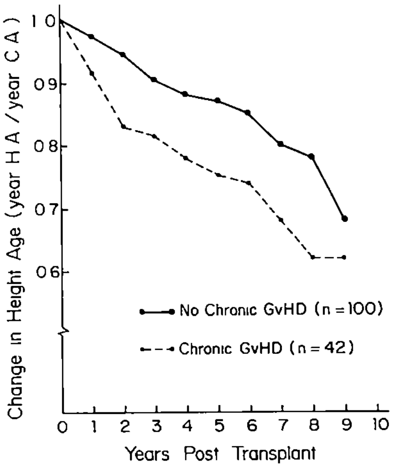

Fig 2. Growth rate following transplant expressed as the change in height age with increasing number of years following total body irradiation. Normal ratio should be 1:00. Solid line represents the change in height age for patients without chronic GVHD and broken line represents the change in height age for patients with chronic GVHD.

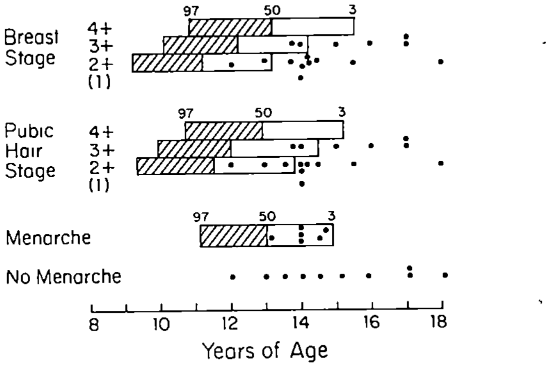

Fig 3. Pubertal developmental stages of 16 girls who were prepubertal at the time of transplant and are currently between 12 and 18 years of age. Solid circles represent their stage of development at their current chronologic age. Open bars represent the 3rd to 97th percentile standards for normal girls.[30]

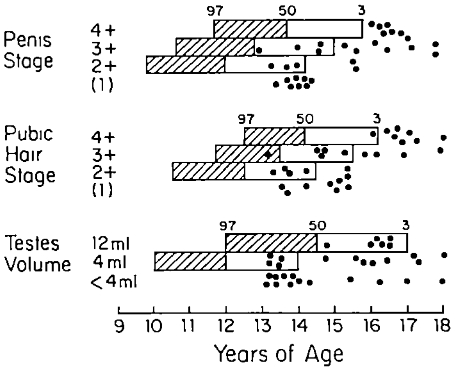

Fig 4. Pubertal developmental stages of 31 boys who were prepubertal at the time of transplant and are currently between 13 and 22 years of age. Solid circles represent their current stage of development. Open bars represent the 3rd to 97th percentile standards for normal boys.[29]

200 ng/dL in 2 who had delayed developmental scores. Elevated LH and FSH levels were present in 10 boys, 4 of whom had testosterone levels <100 ng/dL, 3 of whom had testosterone levels in the 200 to 300 ng/dL range, and 3 had normal testosterone levels. All 10 had delayed development.

Gonadal Function

Forty-four patients were past puberty at the time of transplant as determined by onset of menarche pretransplant for girls and age. All 17 girls had amenorrhea, elevated LH and FSH levels, and decreased estradiol levels for the first 2 years after transplant. Among 14 girls now between 3 and 14 years after transplant, 4 have demonstrated recovery of ovarian function with LH, FSH, and estradiol levels returning to normal and with return of spontaneous menses at 3, 4, 5, and 5 years after transplant. Marrow transplant preparation was 10.0 Gy single exposure TBI for 2 and 12.0 Gy fractionated TBI for 2 of these girls. One of these 4 became pregnant and had an elective abortion, but by 10 years posttransplant she redeveloped amenorrhea and elevated LH and FSH levels. Cyclic hormone supplementation has been given to all of the remaining 10 girls, all of whom continue to have elevated levels of LH and FSH. Gonadotropin and testosterone levels were obtained on 25 of 27 boys. Ten of 25 had elevated LH levels, 23 had elevated FSH levels, and testosterone was normal for 26. Semen analysis in 4 boys demonstrated azoospermia.

DISCUSSION

Multiagent chemotherapy and radiotherapy given to children for treatment of hematologic malignancies are associated with delayed complications that occur months to years later. As these children are surviving longer, the delayed sequelae are becoming increasingly apparent.[37] Late adverse effects in multiple organ systems are, in general, related to particular treatment regimens. Prior to referral for transplant the children in the present study had received chemotherapy with or without radiotherapy. Since none of these children had growth and development evaluations performed before transplant, the specific treatment regimen responsible for the observed abnormalities cannot be determined with certainty.

In the current study asymptomatic compensated hypothyroidism, overt hypothyroidism, hyperthyroxenemia, and a thyroid adenoma were all observed after transplant. Others have also observed a similar incidence of thyroid function abnormalities after TBI in clinically asymptomatic patients.[11] The incidence and type of abnormalities observed after TBI are quite similar to those that have been reported following head and neck irradiation in children, but differ from the much lower incidence reported after cranial irradiation alone.[12,13,38-43] An explanation for the hyperthyroxenemia present in our patients is not readily apparent, but may be due to thyroid damage after TBI. The possibility of thyroid binding globulin abnormalities was not evaluated, however none of the patients with elevated T_4 were taking medication or had liver disease at the time studies were obtained. Only one thyroid neoplasm was found, but the median time of observation is only 4 years after TBI. The increased incidence of thyroid neoplasms and benign thyroid nodules in children after irradiation to the neck has a latent period from 3 to 30 years (median 11 years).[41,42,44,45] Untreated compensated hypothyroidism after irradiation has been suggested as an important factor in the development of neoplastic thyroid disease.[40] Few of our patients with hypothyroidism have been treated with hormone supplementation which may be a factor in future development of thyroid tumors and also may be a factor in their decreased growth. Children surviving after TBI need to be evaluated for development of thyroid dysfunction, have hypothyroidism treated, and be evaluated for thyroid nodules or malignancies.

The long-term effects of irradiation to adrenal glands have not been previously reported, but subnormal stimulated cortisol levels have been observed following central nervous system prophylaxis.[13] The single-dose metapyrone test was selected to evaluate the patients in this study because of its ease of administration. The differentiation of primary or secondary adrenal insufficiency due to adrenocorticotropin hormone deficiency was not explored. Although none of our patients have become symptomatic, their ability to handle major stressful situations, such as surgery, is unknown.

GH deficiency following central nervous system irradiation has been variable, and the effect of irradiation has been correlated with total dosage received.[12-17] The differing results of GH production in children receiving similar doses of irradiation may be due to use of various GH stimuli and/or variation in time intervals between irradiation and GH determination. Both subnormal and normal growth rates have been reported. In the present study, decreased growth rates and GH deficiency were observed after TBI. Previous cranial irradiation, type of TBI administered, and chronic GVHD appear to be the major factors contributing to decreased growth. Since none of the children had GH testing prior to TBI and only 30% had testing after TBI, the actual incidence and time of appearance of GH deficiency cannot be determined.

The severity of epiphyseal, metaphyseal, and diaphyseal injury after irradiation to developing bone appears to increase with increasing radiation dose, lengthening postirradiation interval, and younger age at the time of treatment.[48] Spinal growth suppression occurs after 15 to 44 Gy irradiation to the entire length of spine.[48,49] Children in the present study all received TBI and all had decreased growth rates. The type of TBI administered did not appear to affect height until 3 or more years after transplant when those who received fractionated TBI were observed to grow significantly taller than those who received single exposure TBI.

The acute growth suppressive effects of glucocorticoids would explain the deceleration of linear growth in most chronic GVHD patients. Failure to achieve catch-up growth after completion of treatment and equally decreased growth rates of the patients with chronic GVHD who did not receive treatment suggest that factors other than steroids contributed to their decreased growth.[18,50–53] Since chronic GVHD often involves the liver, synthesis of somatomedins may be diminished and contribute to decreased growth rates.[18,19] Prospective studies utilizing immunosuppressive therapy other than glucocorticoids and further study of GH secretion and somatomedin levels may provide a more complete understanding of the influence of chronic GVHD and/or its treatment on growth after transplantation.

In patients who have received TBI, which is known to cause gonadal failure in postpubertal patients,[9,10] it is reasonable to consider puberty in terms of development of secondary sex characteristics.[29,30] Data from studies of long-term survivors of childhood leukemia who were treated according to conventional protocols indicate an excellent prognosis for normal pubertal progression and gonadal function.[54–56] The current evaluation shows that the majority of children, prepubertal at the time of TBI administration, have a delay in the onset and developmental progression of secondary sexual characteristics due to hypergonadotropic hypogonadism. Their low levels of sex hormones may be a major contributing factor in their lack of an increase in growth velocity during adolescence since normally the increasing sex hormone levels during puberty contribute to growth by increasing growth hormone production.[57,58] The observation that a few girls did achieve menarche and that some boys had normal LH, FSH, and testosterone suggests that irradiation to the prepubertal ovary and testes may not always result in irreversible damage and thus, may not always result in sterility. It is reasonable, however, to consider the use of appropriate sex hormone therapy for children with hypergonadotropic hypogonadism after TBI to promote development of secondary sexual characteristics and perhaps improve growth rates.

Total body irradiation has been shown to produce primary gonadal failure for up to 3 to 6 years in nearly all patients postpubertal at the time of transplant.[9,10] A rare patient has recovered gonadal function. Among the postpubertal patients in the current study, 4 of 17 girls had recovery of ovarian function which was transient in 1. None of the boys have had return of spermatogenesis but Leydig cell function appears adequate in most.

This study shows that multiple endocrine abnormalities may occur following marrow transplantation after chemotherapy and TBI. Although the marrow transplant preparative regimen may not be the treatment modality solely responsible for inducing the observed abnormalities, careful attention to the endocrine system and long-term evaluations of these children are necessary in order to initiate appropriate hormone supplementation which may result in improved growth and development.

REFERENCES

1 Sanders JE, Buckner CD, Stewart P, Thomas ED. Successful treatment of juvenile chronic granulocytic leukemia with marrow transplantation. Pediatrics 63.44, 1979

2. Sanders JE, Flournoy N, Thomas ED, Buckner CD, Lum LG, Clift RA, Appelbaum FR, Sullivan KM, Stewart P, Deeg HJ, Doney K, Storb R: Marrow transplant experience in children with acute lymphoblastic leukemia· An analysis of factors associated with survival, relapse and graft-versus-host disease Med Pediatr Oncol 13:165, 1985

3. Sanders JE, Thomas ED, Buckner CD, Flournoy N, Stewart PS, Clift RA, Lum L, Bensinger WI, Storb R, Appelbaum FR, Sullivan KM: Marrow transplantation for children in first remission of acute nonlymphoblastic leukemia: An update. Blood 66.460, 1985

4. Thomas ED, Clift RA, Fefer A, Appelbaum FR, Beatty P, Bensinger WI, Buckner CD, Cheever MA, Deeg HJ, Doney K, Flournoy N, Greenberg P, Hansen JA, Martin P, McGuffin R, Ramberg R, Sanders JE, Singer J, Stewart P, Storb R, Sullivan K, Weiden PL, Witherspoon R: Marrow transplantation for the treatment of chronic myelogenous leukemia. Ann Intern Med 104:155, 1986

5. Woods WG, Nesbit ME, Ramsay NKC, Krivit W, Kim TH, Goldman A, McGlave PB, Kersey JH: Intensive therapy followed by bone marrow transplantation for patients with acute lymphocytic leukemia in second or subsequent remission Determination of prognostic factors (a report from the University of Minnesota Bone Marrow Transplantation Team). Blood 61:1182, 1983

6. Dinsmore R, Kirkpatrick D, Flomenberg N, Gulati S, Kapoor N, Shank B, Reid A, Groshen S, O'Reilly RJ: Allogeneic bone marrow transplantation for patients with acute lymphoblastic leukemia Blood 62:381, 1983

7. Kersey JH, Ramsay NKC, Kim T, McGlave P, Krivit W, Levitt S, Filipovich A, Woods W, O'Leary M, Coccia P, Nesbit ME: Allogeneic bone marrow transplantation in acute nonlymphocytic leukemia: A pilot study. Blood 60:400, 1982

8. Johnson FL, Thomas ED, Clark BS, Chard RL, Hartmann JR, Storb R: A comparison of marrow transplantation with chemotherapy for children with acute lymphoblastic leukemia in second or subsequent remission. N Engl J Med 305:846, 1981

9. Sanders JE, Buckner CD, Leonard JM, Sullivan KM, Witherspoon RP, Deeg HJ, Storb R, Thomas ED. Late effects on gonadal function of cyclophosphamide, total-body irradiation, and marrow transplantation. Transplantation 36:252, 1983

10 Sklar CA, Kim TH, Williamson JF, Ramsay NK: Ovarian function after successful bone marrow transplantation in postmenarcheal females Med Pediatr Oncol 11:361, 1983

11. Sklar CA, Kim TH, Ramsay NK: Thyroid dysfunction among long-term survivors of bone marrow transplantation. Am J Med 73:688, 1982

12 Richards GE, Wara WM, Grumbach MM, Kaplan SL,

Sheline GE, Conte FA: Delayed onset of hypopituitarism: Sequelae of therapeutic irradiation of central nervous system, eye, and middle ear tumors. J Pediatr 89 553, 1976

13. Oliff A, Bode U, Bercu BB, Di Chiro G, Graves V, Poplack DG. Hypothalamic-pituitary dysfunction following CNS prophylaxis in acute lymphocytic leukemia Correlation with CT scan abnormalities. Med Pediatr Oncol 7.141, 1979

14. Romshe CA, Zipf WB, Miser A, Miser J, Sotos JF, Newton WA. Evaluation of growth hormone release and human growth hormone treatment in children with cranial irradiation-associated short stature. J Pediatr 104:177, 1984

15. Shalet SM, Price DA, Beardwell CG, Morris Jones PH, Pearson D: Normal growth despite abnormalities of growth hormone secretion in children treated for acute leukemia. J Pediatr 94:719, 1979

16 Shalet SM, Beardwell CG, Pearson D, Morris Jones PH: The effect of varying doses of cerebral irradiation on growth hormone production in childhood. Clin Endocrinol 5·287, 1976

17. Elders MJ: Differential effects of cranial radiation on growth hormone response to arginine and insulin infusion J Pediatr 92:754, 1978

18 Unterman TG, Phillips LS: Glucocorticoid effects on somatomedins and somatomedin inhibitors. J Clin Endocrinol Metab 61 618, 1985

19. Sullivan KM, Storb R, Witherspoon R, Shulman H, Deeg HJ, Schubert M, Doney K, Appelbaum F, Tsoi M-S, Sale G, Sanders J, McDonald G, Thomas ED: Biology and treatment of chronic graft-versus-host disease, in Gale RP (ed): Recent Advances in Bone Marrow Transplantation. New York, Alan R Liss, 1983, p 331

20. Thomas ED, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, Lerner KG, Glucksberg H, Buckner CD: Bone-marrow transplantation. N Engl J Med 292·832, 895, 1975

21 Badger C, Buckner CD, Thomas ED, Clift RA, Sanders JE, Stewart PS, Storb R, Sullivan KM, Shulman H, Flournoy N: Allogeneic marrow transplantation for acute leukemia in relapse. Leuk Res 6.383, 1982

22. Lam W-C, Order SE, Thomas ED· Uniformity and standardization of single and opposing cobalt 60 sources for total body irradiation. Int J Radiat Oncol Biol Phys 6:245, 1980

23. Thomas ED, Storb R· Technique for human marrow grafting. Blood 36·507, 1970

24. Deeg HJ, Storb R, Thomas ED, Flournoy N, Kennedy MS, Banaji M, Appelbaum FR, Bensinger WI, Buckner CD, Clift RA, Doney K, Fefer A, McGuffin R, Sanders JE, Singer J, Stewart P, Sullivan KM, Witherspoon RP: Cyclosporine as prophylaxis for graft-versus-host disease A randomized study in patients undergoing marrow transplantation for acute nonlymphoblastic leukemia. Blood 65.1325, 1985

25. Thompson CB, Sanders JE, Flournoy N, Buckner CD, Thomas ED: The risks of central nervous system relapse and leukoencephalopathy in patients receiving marrow transplants for acute leukemia. Blood 67:195, 1986

26. Sullivan KM, Shulman HM, Weiden PL, Storb R, Tsoi MS, Thomas ED: The spectrum of chronic graft-vs-host disease in man, in Gale RP, Fox CF (eds)· Biology of Bone Marrow Transplantation. Orlando, Fla, Academic Press, 1980, p 69

27. Doney KC, Weiden PL, Storb R, Thomas ED. Treatment of graft-versus-host disease in human allogeneic marrow graft recipients: A randomized trial comparing antithymocyte globulin and corticosteroids. Am J Hematol 11:1, 1981

28. Deeg HJ, Loughran TP Jr, Storb R, Kennedy MS, Sullivan KM, Doney K, Appelbaum FR, Thomas ED: Treatment of human acute graft-versus-host disease with antithymocyte globulin and cyclosporine with or without methylprednisolone Transplantation 40:162, 1985

29. Tanner JM, Whitehouse RH: Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty Arch Dis Child 51:170, 1976

30. Marshall WA, Tanner JM. Variations in pattern of pubertal changes in girls. Arch Dis Child 44:291, 1969

31. Greulich W, Pyle SL. A Radiographic Standard of Reference for the Growing Hand and Wrist. Chicago, Year Book Medical Publishers, 1971

32. National Center for Health Statistics. NCHS Growth Charts: Monthly Vital Statistics Report Vol 25, No 3, Supp (HRA). Rockville, Md, Health Resources Administration, 1976, pp 76–1120

33. Fleiss J: Statistical Methods for Rates and Proportions New York, John Wiley, 1973

34 Kalbfleisch JD, Prentice RL: The Statistical Analysis of Failure Time Data. New York, John Wiley, 1980

35. Cox DR: Regression models and life-tables (with discussions) J Roy Stat Soc B 34:187, 1972

36 Steele RGD, Torrie JH: Principles and Procedures of Statistics. New York, McGraw-Hill, 1960

37. Meadows AT, Silber J: Delayed consequences of therapy for childhood cancer Cancer Journal for Clinicians 35:271, 1985

38 Prentice RL, Kato H, Yoshimoto K, Mason M: Radiation exposure and thyroid cancer incidence among Hiroshima and Nagasaki residents. Natl Cancer Inst Monogr 62 207, 1982

39. Schneider AB, Bekerman C, Favus M, Frohman LA, Gonzalez C, Ryo UY, Sievertsen G, Pinsky S: Continuing occurrence of thyroid nodules after head and neck irradiation Relation to plasma thyroglobulin concentration. Ann Intern Med 94:176, 1981

40 Razack MS, Sako K, Shimaoka K, Getaz EP, Rao U, Parthasarathy KL· Radiation-associated thyroid carcinoma. J Surg Oncol 14:287, 1980

41. Fleming ID, Black TL, Thompson EI, Pratt C, Rao B, Hustu O Thyroid dysfunction and neoplasia in children receiving neck irradiation for cancer. Cancer 55:1190, 1985

42. Green DM, Brecher ML, Yakar D, Blumenson LE, Lindsay AN, Voohess ML, MacGillivray M, Freeman AI: Thyroid function in pediatric patients after neck irradiation for Hodgkin disease. Med Pediatr Oncol 8.127, 1980

43 Glatstein E, McHardy-Young S, Brast N, Eltringham JR, Kriss JP: Alterations in serum thyrotropin (TSH) and thyroid function following radiotherapy in patients with malignant lymphoma J Clin Endocrinol Metab 32·833, 1971

44. Maxon HR, Thomas SR, Saenger EL, Buncher CR, Kereiakes JG: Ionizing irradiation and the induction of clinically significant disease in the human thyroid gland. Am J Med 63:967, 1977

45. Raventos A, Winship T. The latent interval for thyroid cancer following irradiation. Radiology 83:501, 1964

46 Robison LL, Nesbit ME Jr, Sather HN, Meadows AT, Ortega JA, Hammond GD: Height of children successfully treated for acute lymphoblastic leukemia: A report from the Late Effects Study Committee of Children's Cancer Study Group. Med Pediatr Oncol 13·14, 1985

47 Berry DH, Elders MJ, Crist W, Land V, Lui V, Sexauer AC, Dickinson L: Growth in children with acute lymphocytic leukemia: A Pediatric Oncology Group Study. Med Pediatr Oncol 11.39, 1983

48 Parker RG, Berry HC: Late effects of therapeutic irradiation on the skeleton and bone marrow. Cancer 37·1162, 1976 (suppl 2)

49. Probert JC, Parker BR: The effects of radiation therapy on bone growth. Radiology 114:155, 1975

50. Loeb JN: Corticosteroids and growth. N Engl J Med 295:547, 1976

51. Blodgett FM, Burgin L, Iezzoni D, Gribetz D, Talbott NB: Effects of prolonged cortisone therapy on the statural growth, skeletal maturation, and metabolic status of children. N Engl J Med 254:636, 1956

52. McEnery PT, Gonzalez LL, Martin LW, West CD: Growth and development of children with renal transplants. Use of alternate day steroid therapy. J Pediatr 83.806, 1973

53. Morris HG, Jorgensen JR, Jenkins SA: Plasma growth hormone concentration in corticosteroid-treated children. J Clin Invest 47:427, 1968

54 Shalet SM, Hann IM, Lendon M, Morris Jones PH, Beardwell CG: Testicular function after combination chemotherapy in childhood for acute lymphoblastic leukaemia. Arch Dis Child 56:275, 1981

55. Siris ES, Leventhal BG, Vaitukaitis JL: Effects of childhood leukemia and chemotherapy on puberty and reproductive function in girls. N Engl J Med 294.1143, 1976

56. Blatt J, Poplack DG, Sherins RJ: Testicular function in boys after chemotherapy for acute lymphoblastic leukemia. N Engl J Med 304:1121, 1981

57 Martin LG, Clark JW, Connor TB: Growth hormone secretion enhanced by androgens. J Clin Endocrinol 28:425, 1968

58. Finkelstein J, Roffwarg HP, Boyar RM, Krema J, Hellman L: Age-related change in the 24-hour spontaneous secretion of growth hormone. J Clin Endocrinol Metab 35:665, 1972

Deoxycytidine Stimulates the In Vitro Growth of Normal CFU-GM and Reverses the Negative Regulatory Effects of Acidic Isoferritin and Prostaglandin E_1

By Kapil Bhalla, John Cole, William MacLaughlin, Michael Baker, Zalmen Arlin, Gary Graham, and Steven Grant

We have examined the effect of supraphysiologic concentrations of the naturally occurring nucleoside deoxycytidine (dCyd) on the in vitro growth of normal (CFU-GM) and leukemic (L-CFU) myeloid progenitor cells. Bone marrow samples obtained from 34 consecutive patients undergoing routine diagnostic bone marrow aspirations for nonmalignant hematologic disorders exhibited nearly a twofold increment in CFU-GM when continuously cultured in the presence of 10^{-4} mol/L dCyd. Higher dCyd concentrations were associated with a smaller degree of enhancement of colony formation. In contrast, the growth of leukemic blast progenitors obtained from patients with acute nonlymphocytic leukemia were not enhanced by any of the dCyd concentrations tested. Coadministration of 10^{-3} mol/L tetrahydrouridine (THU), a cytidine deaminase inhibitor, failed to alter the relative inability of dCyd to enhance L-CFU colony growth. The stimulatory effect of dCyd on normal CFU-GM was not mediated by the adherent mononuclear cell population of the marrow, nor was it restricted to the subpopulation of CFU-GM in S phase at the time of initial exposure. Moreover, treatment of normal bone marrow cells with dCyd at concentrations ranging from 10^{-8} to 5×10^{-3} mol/L for 24 hours had only a minor effect on the fraction of CFU-GM in S phase. Coadministration of 10^{-4} mol/L dCyd was able to reverse the inhibitory effects of several putative regulators of normal myelopoiesis, including leukemia inhibitory activity (LIA), acidic isoferritins (AIF), and prostaglandin E_1 (PGE_1). Leukemic myeloblasts exposed to 10^{-4} mol/L dCyd exhibited substantial expansion of intracellular pools of dCyd triphosphate (dCTP), demonstrating that inability to metabolize dCyd could not be solely responsible for the absence of growth potentiation in these cells. These studies suggest that supraphysiologic concentrations of dCyd may confer a selective in vitro growth advantage upon normal *v* leukemic myeloid progenitor cells, and may free the former from the inhibitory effects of several potential negative regulators of myelopoiesis.

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DEOXYCYTIDINE (dCyd) is a naturally occurring pyrimidine nucleoside that is present in human plasma at concentrations ranging from 5×10^{-7} to 4×10^{-6} mol/L.[1] It is metabolized intracellularly to its triphosphate derivative, dCTP, which is required by DNA polymerase for ongoing DNA synthesis and cell replication.[2] The enzyme dCyd kinase catalyzes the initial phosphorylation of dCyd to its nucleoside monophosphate derivative, dCMP.[3] This process represents the rate-limiting step in dCyd metabolism.[4] Opposing the phosphorylation of dCyd is the degradative enzyme dCyd deaminase, which converts dCyd to deoxyuridine (dUrd).[5] Alternatively, dCMP may be deaminated to deoxyuridylate (dUMP) by another degradative enzyme, dCMP deaminase.[6] Resting cells maintain low dCTP levels, but exhibit increased activity of dCyd kinase and expand dCTP pools immediately prior to cell division.[7] Since a balanced supply of deoxyribonucleotides is required for DNA synthesis, it is believed that a relative deficiency in intracellular dCTP concentrations may limit DNA replication and thereby modulate growth regulation.[8]

Normal human bone marrow myeloid progenitors (CFU-GM) are known to exhibit a larger growth fraction than their leukemic counterparts[9] and may preferentially utilize the pyrimidine salvage pathway for nucleic acid synthesis.[10] Consequently, it is conceivable that quantitative differences in nucleotide metabolism may exist between normal and neoplastic elements. The aim of these studies was to determine whether such differences might be exploited in order to confer an in vitro growth advantage on normal cells. Specifically, we have examined the effect of supraphysiologic concentrations of dCyd on the soft agar growth of normal and leukemic myeloid progenitors. An additional goal was to determine whether high concentrations of dCyd might reverse the growth inhibitory effects toward normal cells of several putative regulators of granulopoiesis, including leukemia inhibitory activity (LIA),[11] acidic isoferritins (AIF),[12] and prostaglandin E_1 (PGE_1).[13] Our results suggest that supraphysiologic concentrations of dCyd may preferentially stimulate the in vitro growth of normal CFU-GM, and may overcome the inhibitory effects of potentially important physiologic modulators of myelopoiesis.

MATERIALS AND METHODS

Drugs and chemicals dCyd, thymidine (dThd), hydroxyurea (HU), indomethacin, and PGE_1 were purchased from Sigma Chemicals (St Louis, Mo). Tetrahydrouridine (THU) was provided by Dr Kenneth Paull, Drug Development Branch, NCI. AIF (pH < 5.0 after isoelectric focusing) was prepared for the spleen of a patient with chronic myelogenous leukemia This isoferritin (94% AIF) as determined by radioimmunoassay,[12] was kindly provided by Dr H Broxmeyer, University of Indiana School of Medicine. [^{3}H]Thymidine (50 Ci/mmol/L) was purchased from Amersham Radiochemicals (Springfield, Ill). Drugs were stored as dry powders at −20 °C and reconstituted in RPMI media immediately prior to use. The

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Culture of normal human bone marrow granulocyte-macrophage progenitors. Human bone marrow myeloid progenitors were cultured by a previously described double-layer soft agar cloning method.[14] Samples were obtained with informed consent from 34 consecutive patients undergoing routine diagnostic bone marrow aspirations for nonneoplastic hematologic disorders These disorders consisted principally of iron deficiency anemia, immune thrombocytopenias, and the anemia of chronic disease. Specifically excluded were bone marrow aspirates from patients with myelodysplastic syndromes, drug-induced leucopenias, myelophthisic processes, acquired immune deficiency syndrome, or any other qualitative or quantitative disorder of myelopoiesis. After isolation of mononuclear cells, appropriate concentrations of dCyd were added prior to plating in soft agar and McCoy's 5a medium. GCT-conditioned media (10% v/v; GIBCO, Grand Island, NY) was used as a source of colony-stimulating activity. Additionally, in a separate set of experiments, cells were plated in the continuous presence of 10^{-3} mol/L THU (a concentration that maximally inhibits the deamination of dCyd)[15] alone, and in conjunction with 10^{-4} mol/L dCyd. Alternatively, bone marrow mononuclear cells were treated for 24 hours with dCyd concentrations ranging from 10^{-6} to 5×10^{-3} mol/L, washed twice with McCoy's medium, and then plated in soft agar as above. In separate experiments, adherent bone marrow cells containing 90% or greater monocytes and macrophages were removed by incubation in tissue culture flasks in McCoy's medium with 10% fetal calf serum for one hour.[16] Nonadherent mononuclear bone marrow cells were plated with various dCyd concentrations as described above. At the end of seven days incubation in a 37 °C, 5% CO_2 fully humidified incubator (NAPCO, Portland, Or), colonies, consisting of groups of 50 or more cells, were scored with an inverted microscope. The effect of various concentrations of dCyd on the fraction of clonogenic CFU-GM in S phase was examined utilizing a previously described [^{3}H]dThd suicide technique [17] Alternatively, the effect of dCyd on non–S phase CFU-GM was assessed by culturing cells in the presence of dCyd following a one-hour exposure to 10^{-3} mol/L HU.[16] In additional experiments, bone marrow mononuclear cells from 6 marrow aspirates were cultured in a manner identical to that for L-CFU (vide infra). Cells were depleted of T lymphocytes by rosetting with sheep red blood cells and plated in soft agar in the presence of either 10% GCT conditioned media or PHA-LCM[18] as a source of colony-stimulating activity. Colony formation was determined as described above.

Culture of leukemic blast progenitors. Leukemic myeloblasts were cultured in soft agar by minor modifications of a previously described technique.[19] T lymphocyte–depleted mononuclear cells obtained from bone marrow or peripheral blood samples from patients with acute nonlymphocytic leukemia were plated at 2×10^5 cells/mL in soft agar utilizing the bilayer soft agar method previously described for normal CFU-GM.[14] The effect of continuous exposure to various conditions of dCyd alone, 10^{-3} mol/L THU alone, and the two agents in combination upon L-CFU colony formation was examined by incorporating the drugs in the top layer of soft agar. PHA leukocyte–conditioned medium (10% v/v) was used as a source of colony-stimulating activity.[20] Plates were incubated at 37 °C, 5% CO_2 fully humidified incubator for seven days and colonies, consisting of groups of 20 or more blastlike cells, were scored with an inverted microscope (Olympus, New Hyde Park, NY).

"Crude" leukemia inhibitory activity, acidic isoferritin, and prostaglandin E_1 studies. Cells were cultured in the presence of 10^{-6} or 10^{-4} mol/L dCyd, along with each negative regulator of myelopoiesis Cell-free "crude" LIA was prepared utilizing the method described by Broxmeyer et al.[11] Maximal inhibitory concentrations of crude LIA (10% v/v), AIF (10^{-9} mol/L), or PGE_1 (10^{-6} mol/L) were added to each plate. In the case of PGE_1, indomethacin (10^{-6} mol/L) was added to the plates to prevent endogenous prostaglandin production by monocytes and macrophages,[16] and appropriate controls assessed.

Deoxyribonucleotide determinations. Normal bone marrow mononuclear cells and leukemic myeloblasts were obtained as described previously, suspended in McCoy's 5a medium containing 10% fetal calf serum, and placed in 25 cm^2 tissue culture flasks along with varying concentrations of dCyd with and without 10^{-3} mol/L THU. After 18 hours incubation in a 37 °C, 5% CO_2 incubator, the cells were centrifuged, the cell pellet precipitated with 0.75 mL of cold 0.5N $HClO_4$, and the supernatant neutralized with cold 4N KOH. The $KClO_4$ precipitate was removed and the neutralized acid soluble extracts assayed for dCTP levels utilizing the DNA polymerase assay of Grindey and Kinahan, described elsewhere in detail.[21]

Statistical methods. Significant differences between experiment groups were assessed utilizing the Student's *t* test for paired or unpaired observations.

RESULTS

The effects of increasing concentrations of dCyd on CFU-GM and leukemic myeloid progenitor (L-CFU) colonies in soft agar are shown in Fig 1. At physiologic concentrations of dCyd in the culture medium (10^{-6} mol/L), no stimulation in colony formation was observed. Exposure to 10^{-4} mol/L dCyd led to a significant increase in CFU-GM colony formation to 193 ± 11.5% of control ($P < 0.001$). At higher concentrations of dCyd, a decline in colony formation to control values was noted. For example, dCyd levels of 10^{-3} and 5×10^{-3} mol/L were associated with 139 ± 8.5 and 96.5 ± 4.6% of control colony growth, respectively. The degree of stimulation varied among different bone marrow samples. However, only 3 our of 34 bone marrow samples failed to exhibit significant enhancement of colony growth following exposure to 10^{-4} mol/L dCyd concentration, and no sample exhibited inhibition. A comparable degree of

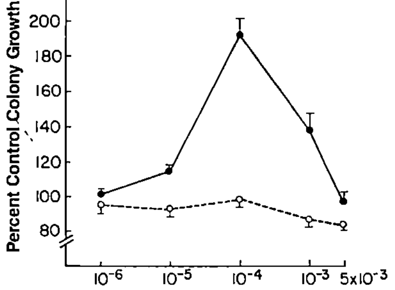

Fig 1. The effect of various concentrations of dCyd on the colony growth of CFU-GM and L-CFU: seven-day CFU-GM (——); seven-day L-CFU (-----). The ordinate is expressed as the percentage of control colony formation. Each point represents the mean value ± SE for at least 11 separate experiments. The number of separate samples assessed for each dCyd concentration ranged from 11 (10^{-5} mol/L) to 34 (10^{-4} mol/L) for CFU-GM and 11 (10^{-6} mol/L) to 30 (10^{-4} mol/L) for L-CFU.

stimulation was observed when 10d CFU-GM were scored instead of day 7 colonies (10 samples). A shorter dCyd exposure interval (24 hours) immediately prior to culture also resulted in a significant growth enhancement (201.7 ± 7.1% of control values; not shown). The degree of enhancement of colony formation by dCyd was not altered either by depleting the mononuclear cell population of T lymphocytes or by utilizing PHA-LCM as a source of colony-stimulating activity. For example, when cells from 6 samples were plated under these conditions, the mean increment in colony formation in the presence of 10^{-4} mol/L dCyd was 198.6 ± 19.1% of control. This value was not significantly different from that obtained with dCyd-treated cells plated under standard conditions. A loss of expression of Ia-antigen on CFU-GM has been reported when the latter is incubated in suspension culture at 37 °C for six hours.[13] Treatment of CFU-GM in this manner prior to plating in soft agar with 10^{-4} mol/L dCyd elicited a growth increment of 174.4 ± 12.3% of control (not shown). Removal of the adherent cell population also did not alter the increment in CFU-GM growth (212 ± 6.7% of control values; not shown). In contrast to normal CFU-GM, leukemic myeloid progenitor colony growth was not enhanced by treatment with any of the dCyd concentrations tested (Fig 1). Moreover, at 5×10^{-3} mol/L dCyd, a decrease to 83.9 ± 3.4 of control leukemic colony formation was observed.

To assess what role cytidine deaminase might play in determining the response of normal and leukemic progenitor cells to supraphysiologic concentrations of dCyd, we examined the growth of CFU-GM and L-CFU in the presence of THU and dCyd (Table 1). Continuous exposure to 10^{-3} mol/L THU or 10^{-4} mol/L dCyd alone resulted in little change in the number of L-CFU. Furthermore, coadministration of THU in conjunction with dCyd did not result in enhanced leukemic cell colony formation. With respect to normal CFU-GM, THU did not increase the number of CFU-GM colonies relative to untreated control cells, nor did addition of THU to dCyd increase CFU-GM growth relative to dCyd (10^{-4} mol/L) administered alone.

In separate studies, dCTP pools were determined in leukemic blasts obtained from 14 patients with acute nonlymphocytic leukemia both in the unperturbed state and following exposure to several concentrations of dCyd for 18 hours (not shown). Untreated freshly procured myeloblasts exhibited a mean of 0.52 ± 0.09 pmol dCTP/10^{-6} cells, while exposure to 10^{-4} and 5×10^{-3} mol/L dCyd for 18 hours increased levels to 1.21 ± 0.27 and 3.38 ± 0.98 pmol dCTP/10^{-6} cells, respectively ($P < 0.02$). The effect of THU on intracellular dCTP pool expansion in leukemic myeloblasts exposed to high concentrations of dCyd was also evaluated (Table 2). Leukemic myeloblasts from 4 patients were exposed to 10^{-4} mol/L dCyd for 18 hours in the presence or absence of 10^{-3} mol/L THU and intracellular dCTP levels determined. In the presence of 10^{-4} mol/L dCyd, mean dCTP levels increased from 0.39 ± 0.1 to 0.68 ± 0.14 pmol/10^6 cells. Exposure to THU alone resulted in a smaller increment in dCTP levels (0.57 ± 0.9 pmol/10^6 cells). When cells were exposed to both THU and dCyd, dCTP levels (0.88 ± 0.29 pmol/10^6 cells) were not significantly different from those exposed to dCyd alone ($P > 0.10$). Identical experiments examining dCTP levels were performed on normal bone marrow mononuclear cells separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. The results, in general, were quite similar to those involving leukemic myeloblasts, ie, THU did not enhance the ability of 10^{-4} mol/L dCyd to expand intracellular dCTP pools. It should be noted that for each condition, intracellular dCTP levels were greater for normal bone marrow mononuclear cells than for leukemic myeloblasts. However, these differences did not achieve the level of statistical significance ($P > 0.10$).

Table 1. Effect of Deoxycytidine (dCyd) and Tetrahydrouridine (THU) on the Growth of Normal (CFU-GM) and Leukemic (L-CFU) Myeloid Progenitor Cells

Condition	Percent Control Colony Formation ± SEM	
	CFU-GM	L-CFU
dCyd (10^{-4} mol/L)	193.0 ± 11.5	98.0 ± 2.5
THU (10^{-3} mol/L)	99.6 ± 2.9	96.3 ± 13.0
dCyd (10^{-4} mol/L) + THU (10^{-3} mol/L)	168.8 ± 21.5	83.5 ± 5.3

Bone marrow samples from 12 patients with nonmalignant hematologic disorders and 4 patients with acute nonlymphocytic leukemia were cloned in soft agar in the presence of dCyd (10^{-4} mol/L), THU (10^{-3} mol/L), or both agents simultaneously. At the end of seven days incubation in a 5% CO_2, 37 °C incubator, normal (CFU-GM) and leukemic (L-CFU) myeloid colonies were scored with the aid of an inverted microscope as described in Materials and Methods. Values for each condition are expressed as the percentage of untreated control cell colony formation ± SEM.

The effect of a 24-hour exposure to 10^{-6} and 10^{-4} mol/L dCyd on the percentage of clonogenic CFU-GM in S phase as determined by the thymidine suicide index is shown in Table 3. Neither dCyd concentration increased the percentage of CFU-GM synthesizing DNA. In addition, treatment of cells with 10^{-3} mol/L HU for one hour reduced CFU-GM colony formation to 69.5 ± 1.4% of control. However, addition of 10^{-4} mol/L dCyd significantly increased clonogenicity to 91.3 ± 4.2%, indicating that the effect of dCyd is not restricted to CFU-GM initially in S phase.

The effect of dCyd on the inhibitory activity of some of the known regulators of granulopoiesis is also shown in Table 3. In the absence of dCyd, a significant reduction in CFU-GM growth to 65.7, 64.5, and 55.0% of control values was observed following exposure to crude LIA (10%), AIF (10^{-9} mol/L), and PGE_1 (10^{-6} mol/L), respectively. Coadministration of physiologic concentrations of dCyd (10^{-6} mol/L) did not alter the degree of inhibition by these agents. However, simultaneous treatment with 10^{-4} mol/L dCyd resulted in a significant increment in CFU-GM growth to values exceeding unperturbed control colony formation. The degree of reversal of the growth inhibition by 10^{-4} mol/L dCyd concentrations varied among different bone marrow samples. In general, the abolition of the inhibitory effect of crude LIA, AIF, and PGE_1 was more pronounced in those CFU-GM samples that demonstrated a greater increment in colony growth following treatment with dCyd alone (data not shown).

Table 2. dCTP Pools in Human Leukemic Myeloblasts and Normal Bone Marrow Mononuclear Cells Exposed to Deoxycytidine (dCyd) and/or Tetrahydrouridine (THU)

	N	dCTP Concentrations (pmol dCTP/10^6 cells) Control	dCyd 10^{-4} mol/L	THU 10^{-3} mol/L	dCyd 10^{-4} mol/L + THU 10^{-3} mol/L
Leukemic Blasts					
	1	0.18	0.23	0.51	0.62
	2	0.71	0.98	0.84	1.37
	3	0.42	0.84	0.34	0.88
	4	0.27	0.70	0.61	0.66
Mean ± SEM		0.39 ± 0.10	0.68 ± 0.14	0.57 ± 0.09	0.88 ± 0.29
Normal Bone Marrow Mononuclear Cells					
	1	0.29	1.79	0.68	2.76
	2	0.52	1.21	0.70	1.20
	3	0.59	1.65	1.04	2.45
	4	0.13	0.32	0.15	0.34
Mean ± SEM		0.38 ± 0.09	1.24 ± 0.28	0.64 ± 0.15	1.68 ± 0.48

Leukemic myeloblasts from 4 patients with acute nonlymphocytic leukemia and normal bone marrow mononuclear cells from 4 patients with nonmalignant hematologic disorders were incubated for 18 hours with 10^{-4} mol/L dCyd alone, 10^{-3} mol/L THU alone, and the two agents in combination. At the end of the incubation period, cold ethanol extracts were obtained and dCTP levels determined by the DNA polymerase assay as described in Materials and Methods. Values are expressed as pmol dCTP/10^6 cells.

DISCUSSION

In this report we describe the ability of supraphysiologic concentration (10^{-4} mol/L) of exogenous dCyd to enhance the in vitro growth of normal CFU-GM, but not of leukemic blast progenitors. This stimulatory effect is apparently not mediated through increased elaboration of granulocyte-macrophage colony-stimulating factors (GM-CSF) from monocytes and macrophages in the adherent cell population of bone marrow. Since depletion of the T lymphocyte subpopulation did not reverse the enhancement of colony formation by dCyd, it is unlikely that these cells are implicated in this phenomenon. Previous studies have suggested that putative regulators of myelopoiesis affect only the Ia antigen bearing CFU-GM in S phase.[13] In contrast, our results demonstrate that the dCyd-mediated stimulation of CFU-GM proliferation is not dependent on Ia antigen expression, nor is it limited to CFU-GM in S phase. However, since we have not extracted the Ia antigen–bearing cells from our marrow samples, we cannot rule out the possibility that such cells may play some role in determining the response of CFU-GM to supraphysiologic concentrations of dCyd. Finally, our results indicate that dCyd treatment does not, by itself, increase the percentage of CFU-GM in S phase. Therefore, the twofold increment in CFU-GM colony formation cannot be ascribed to a "recruitment" of myeloid progenitors by dCyd into the DNA synthesis phase of the cell cycle.

An explanation for the inability of dCyd to stimulate the in vitro growth of L-CFU is not readily apparent. One possibility is that leukemic myeloblasts may be highly efficient in their ability to deaminate dCyd by the enzyme cytidine deaminase. Previous studies by Chabner and colleagues have indicated that although cytidine deaminase activity tends to increase with granulocytic maturation, leukemic myeloid progenitors do not exhibit higher levels of cytidine deaminase activity than their normal counterparts at equivalent levels of differentiation.[22] In addition, Coleman and coworkers have reported that dCyd kinase/cytidine deaminase activity ratios in the immature or myeloblast-enriched fractions from normal and leukemic bone marrow samples are quantitatively similar.[23] Results of the present study demonstrate that coadministration of tetrahydrouridine in conjunction with dCyd fails to enhance significantly either the intracellular dCTP pools or the in vitro growth of L-CFU. This suggests

Table 3. Effect of 10^{-6} mol/L and 10^{-4} mol/L dCyd on the Inhibitory Activity of Various Agents on CFU-GM Colony Growth

Conditions	Percent Control CFU-GM Growth Without dCyd	dCyd 10^{-6} mol/L	dCyd 10^{-4} mol/L
Hydroxyurea (10^{-3} mol/L)*	69.5 ± 1.4‡	ND	91.3 ± 4.2§
[^{3}H] Thymidine Suicide Index†	59.0 ± 2.2‡	59.0 ± 3.6	63.0 ± 2.3
"Crude" LIA (10%)	67.7 ± 1.6‡	70.0 ± 3.6	134.0 ± 7.5§
Acidic Isoferritin (10^{-9} mol/L)	64.5 ± 1.5‡	67.4 ± 1.7	123.0 ± 7.8§
Prostaglandin E_1 (10^{-6} mol/L)	55.0 ± 1.6‡	58.2 ± 0.8	121.0 ± 7.6§

ND, not done.

*Cells are incubated for one hour with hydroxyurea, washed, and then plated in the continuous presence of dCyd

†Cells are incubated with [^{3}H] thymidine (50 μCi/mL) for 25 minutes with and without a prior 24-hour incubation with dCyd. They are then washed and plated in the absence of drug.

‡Significantly different from control values ($P < 0.05$).

§Significantly different from colony growth in the absence of dCyd exposure (A) ($P < 0.05$).

that activity of cytidine deaminase in leukemic blasts is not solely responsible for their limited response to dCyd. Despite the results of these in vitro studies, it is possible that deamination may play an important role in determining the pharmacokinetics and biologic effects of dCyd in an intact animal and would have to be taken into consideration in any projected in vivo studies.

The apparent inability of supraphysiologic concentrations of dCyd to stimulate L-CFU growth might also result from a relative impairment in the capacity of leukemic cells to expand dCTP pools in response to exogenous dCyd. However, our studies demonstrate that leukemic blasts are capable of increasing intracellular dCTP pools significantly under these conditions and that this phenomenon is not associated with potentiation of colony formation. Other potential explanations include the possibilities that leukemic cells may actively degrade dCyd nucleotides utilizing enzymes such as 5′-nucleotidase[24] or higher dephosphorylases,[25] or that intracellular dCTP levels might be less limiting for the in vitro growth of leukemic cells than for normal myeloid progenitor cells. Finally, it is conceivable that biochemical perturbations in normal and leukemic cell populations as a whole do not reflect events occurring in the small subset of clonogenic cells. For example, in our studies, intracellular levels of dCTP under various conditions tended to be greater for normal mononuclear cells than for leukemic myeloblasts, although the differences did not achieve statistical significance. However, it is known that the normal bone marrow mononuclear cell population separated by Ficoll-Paque density gradient centrifugation is heterogeneous and does not represent a pure population of clonogenic cells.[26] Consequently, direct comparisons of biochemical differences between normal and leukemic bone marrow cells (eg, dCyd kinase or cytidine deaminase activity, perturbations in dCTP pools) might not predict the behavior of the small subset of myeloid progenitors. It is likely that advances in the purification of normal and leukemic stem cells will be necessary before definitive biochemical differences can be characterized.

AIF have been identified as the mediators of the inhibitory effects of LIA on CFU-GM growth.[12] Both "crude" LIA and AIF are relatively incapable of inhibiting the in vitro proliferation of blast cells from patients with acute nonlymphocytic leukemia,[27] leading to the hypothesis that AIF confers a proliferative advantage of leukemic blasts over normal myeloid progenitors. Our study demonstrates that supraphysiologic concentrations of dCyd (10^{-4} mol/L) in the culture medium reverses the inhibitory effect of AIF on CFU-GM. This effect was also observed with respect to PGE_1, which is produced by monocytes and macrophages, and suppresses colony formation by cycling, Ia antigen positive, macrophage progenitor cells.[13] Whether dCyd directly antagonizes the activity of these factors or stimulates subpopulations of cells immune to their actions remains to be established.

The mechanism(s) underlying in vivo suppression of normal hematopoiesis in leukemia are not clearly understood. In vitro studies have recognized several growth promoters for normal hematopoiesis such as interleukin 3, GM-CSF, and colony-stimulating activity derived from fibroblasts.[28] On the other hand, the inhibitory influences of AIF, PGE_1, and lactoferrin are also well characterized.[12,13,29] It is not currently known whether it is inadequate expression of growth factors or enhanced manifestation of the inhibitors that favors leukemic progenitor cell growth. Our study provides in vitro evidence that supraphysiologic concentrations of dCyd may protect normal myeloid progenitors from inhibitory regulators and may directly stimulate their proliferation. It also raises the possibility that modulation of exogenous dCyd concentrations may confer a selective growth advantage on normal myelopoiesis. In view of the potential therapeutic benefits of dCyd in leukemia, animal studies designed to assess the in vivo relevance of these findings appear warranted.

REFERENCES

1. Danhauser LL, Rusturn YM. Effect of thymidine on the toxicity, antitumor activity, and metabolism of 1-B-D-arabinofuranosylcytosine in rats bearing a chemically induced colon carcinoma Cancer Res 40 1274, 1980

2 Nordenskjord BA, Skoog L, Brown NC, Reichard P. Deoxyribonucleotide pools and DNA synthesis in cultured mouse embryo cells J Biol Chem 245:5360, 1970

3 Momparler RL, Fischer GA Mammalian deoxynucleoside kinases I. Deoxycitine kinase Purification, properties and kinetic studies with cytosine arabinoside. J Biol Chem 243.4298, 1968

4. Plagemann PG, Marz R, Wohlheuter RM: Transport and metabolism of deoxycytidine and 1-B-D-arabinofuranosylcytosine into cultured Novikoff rat hepatoma cells: Relationship to phosphorylation and regulation of triphosphate synthesis. Cancer Res 38.978, 1978

5. Steuart CD, Burke PJ. Cytidine deaminase and the development of resistance of arabinosylcytosine. Nature New Biol 233:109, 1971

6. Maley GF, Maley F: The purification and properties of deoxycytidylate deaminase from chick embryo extracts. J Biol Chem 239·1168, 1964

7 Skoog KL, Nordenskjold BA, Bjursell KG: Deoxyribonucleoside triphosphate pools and DNA synthesis in synchronized hamster cells. Eur J Biochem 33.428, 1973

8. Xeros N· Deoxyriboside control and synchronization of mitoses. Nature 194·68, 1962

9. Lampkin BC, Woods W, Strauss R, Feig S, Higgins G, Bernstein I, D'Angio G, Chard R, Bleyer A, Hammond D: Current status of the biology and treatment of acute nonlymphocytic leukemia in children. Blood 61:215, 1983

10. Rustum YM, Takita H, Gomez G· Metabolic modulation and cellular de novo versus salvage metabolism. Antibiotics Chemother 28·88, 1980

11. Broxmeyer HE, Jacobsen N, Kurland J, Mendelsohn N, Moore MAS: In vitro suppression of normal granulocytic stem cells by inhibitory activity derived from human leukemia cells. J Natl Cancer Inst 69:497, 1978

12 Broxmeyer HE: Bognacki J, Dorner MH, DeSousa M: Identification of leukemia-associated inhibitory activity as acidic isoferritins. A regulatory role for acidic isoferritins in the production of granulocytes and macrophages. J Exp Med 153:1426, 1981

13. Pelus LM, Saletan S, Silver RT, Moore MAS· Expression of

Ia-antigens on normal and chronic myeloid leukemic human granulocyte-macrophage colony forming cells (CFU-GM) is associated with the regulation of cell proliferation by prostaglandin E Blood 59:284, 1982

14. Bhalla K, Nayak R, Grant S: Isolation and characterization of a deoxycytidine kinase-deficient human promyelocytic leukemia cell line highly resistant to 1-B-D-arabinofuranosylcytosine. Cancer Res 44:5029, 1984

15. Chou TC, Arlin Z, Clarkson BD, Philips FS· Metabolism of 1-B-D-arabinofuranosylcytosine in human leukemic cells. Cancer Res 37:3561, 1977

16. Taetle R, Koessler A: Effects of cyclic nucleotides on normal and abnormal human myeloid progenitor proliferation. Cancer Res 40:1223, 1980

17. Preisler H, Shoham D: Comparison of tritiated thymidine labeling and suicide indices in acute nonlymphocytic leukemia Cancer Res 38:3681, 1978

18. Aye MT, Till JE, McCulloch EA: Interacting populations affecting proliferation of leukemic cells in culture. Blood 45:485, 1975

19 Buick RN, Till JE, McCulloch EA Colony assay for blast cells circulating in myeloblastic leukemia. Lancet 1:862, 1977

20. Iscove NN, Till JE, McCulloch EA: The proliferative states of mouse granulopoietic progenitors. Proc Soc Exp Biol Med 143·33, 1970

21. Grindey GB, Wang MC, Kinahan JJ: Thymidine induced perturbations in ribonucleoside and deoxyribonucleoside triphosphate pools in human leukemic CCRF-CEM cells. Mol Pharmacol 16:601, 1979

22. Chabner BA, Johns DG, Coleman CN, Drake JC, Evans WH: Purification and properties of cytidine deaminase from normal and leukemic granulocytes J Clin Invest 53:922, 1974

23. Coleman CN, Stoller RG, Drake JC, Chabner BA: Deoxycytidine kinase: Properties of the enzyme from human leukemic granulocytes. Blood 46:791, 1975

24. Carson DA, Kaye J, Matsumoto S, Seegmiller JE, Thompson L: Biochemical basis for the enhanced toxicity of deoxyribonucleosides toward malignant T cell lines. Proc Natl Acad Sci 76 2430, 1979

25. Abe I, Saito S, Hori K, Suzuki M, Sato H: Role of dephosphorylation in accumulation of 1-B-D-arabinofuranosylcytosine 5'-triphosphate in human lymphoblastic cell lines with reference to their drug sensitivity. Cancer Res 42:2846, 1982

26. Korzumi S, Curt GA, Fine RL, Griffin JD, Chabner BA: Formation of methotrexate polyglutamates in purified myeloid precursor cells and normal bone marrow. J Clin Invest 75:1008, 1985

27. Taetle R: Acidic isoferritins (leukemia associated inhibitory activity) fail to inhibit blast proliferation in acute myelogenous leukemia. Blood 58:653, 1981

28. Dexter TM: The message in the medium. Nature 309.746, 1984

29. Broxmeyer HE; Lactoferrin acts on Ia-like antigen positive subpopulations of human monocytes to inhibit production of colony stimulatory activity in vitro. J Clin Invest 64:1717, 1979

Heterogeneity of Large Granular Lymphocyte Proliferations: Delineation of Two Major Subtypes

By Wing C. Chan, Susan Link, Alison Mawle, Irene Check, Russell K. Brynes, and Elliott F. Winton

Two major types of lymphocytosis of large granular lymphocytes (LGLs) were observed. The proliferating LGLs in each type had distinct immunophenotypes, functional characteristics, and probably belonged to different cell lineages. The more common form (Type A) consisted of cells derived from the T cell lineage and had the T suppressor/cytotoxic phenotype (T11+, T3+, T8+). The expression of the Leu 7 and HLA-DR antigen was variable. These cells did not have natural killer (NK) function but showed a variable degree of antibody-dependent cell-mediated cytotoxic (ADCC) activity. Neutropenia was invariably present and rheumatoid arthritis and autoantibodies were frequent associations. These lymphocytes had many similarities to the major type of LGLs present in normal adult bone marrow. The other type of LGL lymphocytosis (Type B) consisted of cells lacking the antigens T3 and T8 but expressing M1 and NKH1. These cells possessed strong NK and ADCC activity but their cell lineage was not clear. Neutropenia and autoimmune phenomena were not detected. The cytochemical characteristics of the LGL granules from both types of patients were similar but differences in ultrastructure were observed. LGLs from Type B patients proliferated in the presence of Interleukin 2 (IL-2) and 12-0-tetradecanoyl-phorbol-13 acetate (TPA). Significant changes in their basic T11+, T3−, T8− phenotype were not observed. IL-2 and TPA, however, had profound influence on the NK function of the cells with enhancement in the case of IL-2 and marked suppression when stimulated by TPA.

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A UNIQUE population of lymphocytes consisting of cells having abundant cytoplasm with many azurophilic granules has been termed large granular lymphocytes (LGLs). This population of cells constitutes only a minority (mean of approximately 15%) of normal peripheral blood lymphocytes but contains virtually all natural killer (NK) and antibody-dependent cell-mediated cytotoxic (ADCC) activity.[1-3] An increasing number of patients showing a lymphocytosis of peripheral blood and bone marrow LGLs has been recently reported.[4-26] These patients often suffer from associated severe neutropenia and, occasionally, pure red cell aplasia.[6,9,23] The bone marrow generally shows only a mild to moderate increase in lymphocytes, which is insufficient to cause neutropenia by marrow replacement. The lymphocytosis, with few exceptions, is nonprogressive or only slowly progressive over an extended period of time. We have studied 11 patients with LGL lymphocytosis and have observed considerable heterogeneity in the clinicopathologic picture, as well as in the immunophenotypes and in vitro function of the LGLs. These may, however, be grouped into two broad categories that possibly represent derivation from two subtypes of LGLs with different origins and functions.

MATERIALS AND METHODS

All patients in this study had an increased percentage of peripheral blood LGLs (>50% of lymphocytes) The total lymphocyte counts were normal or increased but the LGL counts were all above the range of normal individuals studied (normal range = 87 to 310/cu mm) None of the patients had evidence of an active systemic viral infection within 12 months prior to diagnosis.

Morphologic studies included examination of peripheral blood and bone marrow aspirate smears as well as trephine biopsies obtained from the posterior superior iliac crest.[21] Reticulin stains were performed on trephine biopsy sections.[27] Cytochemical studies included staining for acid phosphatase (ACP), Beta-glucuronidase (BG), alpha-naphthyl acetate esterase (ANAE) activities, and staining for the periodic acid Schiff's (PAS) reaction.[21,28]

Electron microscopy was carried out on blood and/or bone marrow specimens.[22] Immunologic phenotyping was performed on peripheral blood mononuclear cells isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Sheep erythrocyte receptors were demonstrated either by the rosetting technique or by using the monoclonal antibody anti-CD2 (T11).[29] The Fc receptor was demonstrated by rosette formation with rabbit IgG–sensitized ox erythrocytes or with the monoclonal antibody anti-CD16 (anti-Leu 11b) [30] Other monoclonal antibodies used included anti-CD3 (OKT3) for mature T cells, anti-CD4 (anti-Leu 3a or OKT4) for T helper cells, anti-CD8 (anti-Leu 2a or OKT8) for T suppressor cells, anti-Leu 7 for LGLs, NKH1 for LGLs, anti-CD11 (OKM1) for monocytes/granulocytes/LGLs and anti-HLA-DR.[22,31-33] Surface antigens were demonstrated by using monoclonal antibodies in an indirect immunoperoxidase technique[22] or by indirect immunofluorescence and flow cytometry [34]

ADCC and NK activity were measured by a cytotoxicity assay using ^{51}Cr-labeled target cells.[22] The mouse mastocytoma cell line (P815) coated with rabbit IgG (MA Bioproducts, Walkersville, Md) was used in ADCC assays This target cell is sensitive to lymphocyte- but not monocyte-mediated cytotoxicity [35] Human cell lines used in NK assays included K562, the T cell lines KT, HSB-2, MOLT-3, CEM; the Burkitt's lymphoma cell line, RAJI; and the B lymphoblastoid cell lines SB, WIL-2/729 (WIL), GN, and GHC. Percent cytotoxicity was calculated according to the formula:

$$100 \times \frac{\text{Cpm (test wells)} - \text{Cpm (spontaneous release)}}{\text{Cpm (maximum release)} - \text{Cpm (spontaneous release)}} \%$$

Peripheral blood mononuclear cells were cultured in Interleukin 2 (IL-2)–containing medium as follows: Cells were grown in RPMI-1640 with 20% fetal calf serum (FCS), containing 10% IL-2 (Cellular Products, Buffalo, NY), at an initial concentration of 5×10^5 to 1×10^6 cells/mL. When cellular proliferation began, the concentration was then kept below 2×10^5/mL. When sufficient cells were available, cytotoxicity assays and surface marker studies

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were performed. Peripheral blood mononuclear cells were also cultured in 12-0-tetradecanoyl-phorbol-13 acetate (TPA). TPA (Sigma, St. Louis, Mo) was dissolved in acetone at concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mol/L. Each concentration was diluted 1,000-fold with RPMI-1640 containing 10% FCS before being used in culture to achieve final concentrations of 10^{-5} to 10^{-9} mol/L. Cells were adjusted to 5×10^6/mL in TPA-containing medium and 200 μL added to each well of a 96 well plate. Triplicate cultures were set up for each concentration of TPA and incubated in 5% CO_2 at 37 °C for three days or five days. Each well was pulsed with 1 μCi of tritiated thymidine four hours before harvesting, and uptake was determined. TPA-stimulated cells for surface marker determination and cytotoxicity assay were cultured in 24 well plates at 5×10^6 cells/well.

RESULTS

Clinical features. Our patients consisted of 6 males and 5 females with a median age of 57 years at presentation and an age range of 28 to 75 years (Table 1). All were Caucasian. Absolute lymphocytosis ($>4,000/mm^3$) was detected in six while the other patients had an increase in the proportion of LGLs (>50%, normal mean ±1 SD = 12.7 ± 7.1). Significant lymphadenopathy was not detected in any of the patients except in regional lymph nodes draining a site of infection. Slight to moderate splenomegaly was seen in four patients. Patient 8 had marked splenomegaly as well as hepatomegaly but she also suffered from congestive heart failure which contributed significantly to the organomegaly. Hepatomegaly was not observed in the other patients.

A variety of associated disorders were also seen in these patients. Neutropenia ($<1,800/\mu L^3$) was present in nine of the 11 patients. Clinical rheumatoid arthritis was seen in three patients while rheumatoid factor and/or antinuclear antibodies were detected in six. Patient 3 had an abnormal band on protein electrophoresis that was suggestive of immune complex.

Dermal, perianal, and upper respiratory tract infections were the most common complications among the patients with severe neutropenia. Patients 4 and 6 had well-documented episodes of septicemia, which was the cause of death for patient 4. Patient 7 died suddenly at home and autopsy showed multiple ileal ulcers some of which demonstrated transmural necrosis but no perforation. Postmortem blood culture grew gamma streptococci and diptheroids, which probably represented contamination from skin flora. Patient 2 died from massive intestinal infarction 5 years after initial diagnosis.

Three patients were treated with prednisone but only one (patient 7) had a significant partial response with increase of neutrophil count to 500 to 1,000/μL during treatment. Patient 4 underwent splenectomy 2 months after presentation. Her anemia and thrombocytopenia improved but the neutropenia stayed unchanged. Patient 6 had a complete spontaneous remission of his neutropenia, anemia, and lymphocytosis about 13 months after diagnosis and has remained well since. No other patient has demonstrated spontaneous improvement. Patient 1 had a splenectomy following traumatic splenic rupture 3 years before presentation.

Hematologic laboratory examinations. With the exception of patients 1 and 3, all showed neutropenia on hematologic laboratory examination (Table 2). Moderately severe anemia (less than 10 g/dL) was present in three patients (patients 6, 8, 11). Significant thrombocytopenia was found in only patient 4 and this responded completely to splenectomy with the platelet count remaining in the normal range

Table 1. Clinical Features of Patients With LGL Lymphocytosis

Patients	Age/Sex	Splenomegaly	Associated Disease	Complications	Treatment & Response
1	61/M	Splenectomy July 1981	Severe atherosclerotic disease, chronic alcoholism	No infectious complications	No specific treatment
2	57/M	Moderate	Rheumatoid arthritis	Dermal infection, perianal abscesses	No specific treatment; died 5 y after diagnosis from massive intestinal infarction
3	40/F	None	None	No infectious complications	No specific treatment
4	57/F	Moderate	Chronic active hepatitis, systemic hypertension, asthma	Multiple infectious complications	Splenectomy with improvement of anemia and thrombocytopenia
5	68/F	None	Corneal keratolysis, leg ulcers, rheumatoid arthritis	None observed	No specific treatment
6	43/M	Slight	Severe apthous stomatitis	Septicemia	No specific treatment, spontaneous remission
7	28/M	None	Pyoderma gangrenosum	Dermal infections, perirectal abscess	High dose steroid with partial response. Sudden death 4 y after diagnosis
8	66/F	Marked	Cardiac failure, chronic obstructive lung disease	Respiratory tract and ear infection	No specific treatment
9	65/M	None	None	Multiple infectious complications	Prednisone with no response
10	30/M	None	Hematuria, warts	Dermal infections	No specific treatment
11	75/F	Slight	Rheumatoid arthritis, diabetes mellitus	Dermal infections, pneumonitis	Prednisone with minor increase in neutrophils

None of the patients had lymphadenopathy aside from lymph nodes draining sources of infection. There were no skin infiltrates. Treatment refers to attempts at improving the neutropenia.

Table 2. Laboratory Findings of Patients With LGL Lymphocytosis

	Blood				Bone Marrow		Serum	
Patients	Hemoglobin (g/dL)	Neutrophils (/cu mm)	Lymphocytes (/cu mm)	Platelets (/cm mm)	Lymphocytes (%)	Granulopoiesis	Ig	Auto Antibodies
1	14.6	5,940	21,060	300,000	48	Normocellular, maturation orderly	Norm	None detected
2	14.9	384	18,816	114,000	52	Moderately hypocellular, maturation arrest	High IgG IgA	Rheumatoid factor, antinuclear antibody
3	14.8	2,840	12,300	250,000	32.5	Normocellular, maturation orderly	Norm	None detected
4	10.6	0	8,649	53,000	32.5	Hypocellular, maturation arrest	High IgG	Possible immune complex
5	11.0	530	8,480	268,000	26	Normocellular, mild left shift	High IgA	Rheumatoid factor, antinuclear antibody
6	8.7	413	6,408	204,000	37	Normocellular, left shifted	Norm	Antinuclear antibody
7	13.5	384	3,780	261,000	33	Hypocellular, maturation arrest	Norm	None detected
8	9.8	391	2,496	124,000	16	Hypercellular, decreased proportion of segmented forms	High IgG	Rheumatoid factor, antinuclear factor, Cold autoanti-PI
9	10.3	0	1,554	264,000	66.5	Hypocellular, mild left shift with decreased neutrophils	High IgG IgA	Rheumatoid factor, antinuclear antibody
10	13.6	110	952	248,000	34	Hypocellular, left shifted	Norm	Not done
11	8.8	208	936	182,000	35	Normocellular, maturation arrest	High IgG, IgA	Rheumatoid factor, antinuclear antibody

Hemoglobin and platelet count values were obtained at the time of the initial bone marrow biopsy. The neutrophil counts were the lowest recorded values. The lymphocyte counts were those observed at the time of the initial surface marker studies. Minor fluctuations were observed in all the peripheral blood counts during follow-up examinations. For patient 4, the hemoglobin count and platelet count returned to normal after splenectomy accompanied by a progressive increase in lymphocyte count from the 3,000 range to the 8,000 range. Patient 6 underwent a spontaneous remission as described in the text. The lymphocyte count of patients 9 and 10 also decreased and has remained at the lower levels for over 3 years. Granulocyte cellularity was estimated from the marrow cellularity and the differential count as well as from the appearance of the histologic sections. The granulocyte series showed maturation arrest when the number of myelocytes and their precursors were five times that of the later cells.

until the patient's death 7 years later. Patient 8 presented with mild thrombocytopenia which resolved on subsequent determinations. Bone marrow examination showed mild to moderate lymphocytosis. Reticulin fibrosis (Fig 1) was present in ten patients and was unevaluable in one. The fibrosis was severe in one patient, moderate in six, and slight in two. Cellularity and maturation of the granulocytic series were variable. Cellularity was significantly decreased in five patients, slightly increased in one, and normal to slightly decreased in the remaining five patients. A left shift or maturation arrest of the granulocytic series at the myelocyte stage was seen in most marrow specimens from patients with neutropenia. Serum immunoglobulin levels were either normal or increased. Antineutrophil antibodies were determined (kindly performed by Dr G.L. Logue) on the sera of patients 4, 6, 7, 8, and 9 and was borderline increased in patient 4.

Immunophenotypic heterogeneity. Surface marker determinations revealed considerable heterogeneity (Table 3). Two broad groups could, however, be delineated. Nine out of eleven patients had a proliferation of LGLs with the following basic phenotype: T11+, T3+, T8+, T4−. In most of these patients, the cells were also M1−, Leu 7+, Leu11−, and NKH1−. LGLs from patient 4 were unusual in expressing M1 but not Leu 7. Furthermore, 24% of the peripheral blood lymphocytes (PBL) carried the NKH1 antigen. Only 33% of patient 9's PBL expressed Leu 7. There were, however, only 48% T8+ cells and the proportion of T3+, T8+ cells to Leu 7+ cells was comparable to those of patients 6, 7, and 8.

The immunologic phenotype of LGLs from patients 1 and 3 was distinctly different from the former group of patients.

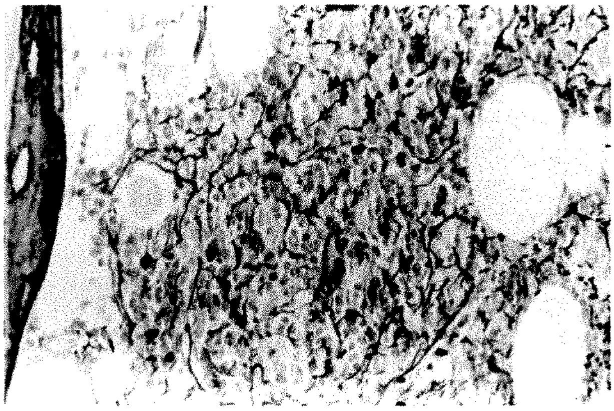

Fig 1. Reticulin stain of the trephine biopsy from patient 1 showing a moderate increase in reticulin fibers. This feature was present in all evaluable biopsies. Snooks reticulin, original magnification ×200.

Table 3. Surface Marker Studies on Peripheral Blood Lymphocytes

		Markers											LGL	
Patients		SIG	ER(T11)	T3	T8	T4	Leu7	OKM1	FcγR	Leu11	NKH-1	HLA-DR	Percent	(Absolute Number)/ cumm
1	3/26/84*		50	10	0	0						81	93	(19586)
	1/3/85†		(98)	6	3	4	5	78		5	87	62		
2	7/80†	2	84	74	68	10		0	64			0	88	(16558)
3	10/30/85†		(98)	17	16	11	3	51		12	68	44	80	(9840)
4	11/9/84†		(92)	92	74	23	2	56		1	24	92	75	(6487)
5	5/14/81*	7	71	79	82	0			30				80	(6784)
6	1/6/82*	3	75	99	99	1	64	0	78			0	79	(5062)
	5/11/82†		(100)	96	90	6	77	3		3	3	8		
7	5/27/81*	2	81	80	72	6	83	0	29, 55			10	79	(2986)
	11/9/82†		(100)	96	90	4	79	2		2	0	0		
8	3/11/85*	15	22(86)		62	20	83					82	87	(2172)
	3/11/85†		(71)	94	64	10	80	2		10	0	31		
9	9/13/85*	23	82	84	50	41							58	(901)
	6/13/85†		(84)	81	48	26	33	12		4	0	20		
10	8/17/82*		83	94	82	16							63	(600)
11	6/8/81*		80		73	32							58	(543)

*Experiment performed using the immunperoxidase method.

†Markers demonstrated by the indirect immunofluorescence method and enumerated using flow cytometry. The number represents the percentage of lymphocytes positive for the marker. Percent LGL was obtained by enumerating the number of granular lymphocytes out of 200 lymphocytes on a Wright's stained blood smear prepared at or close to the time of the corresponding surface marker determination. The dates indicate the time when the test samples were obtained. Results of immunohistochemical and immunofluorescent assays generally corresponded well. The wide discrepancy in the percentage of cells bearing HLA-DR in patient 8 may be partly accounted for by the difference in the methodologies used. The fact that fresh cells were used in the former and cryopreserved cells used in the latter assay and different monoclonal antibodies were employed may also contribute to the difference.

Their LGLs failed to react with anti-T3, T8, Leu 7, and Leu 11. Most of the cells bore T11, M1, and NKH1 antigens.

The number of cells bearing the sheep red blood cell receptor was determined by both the rosetting technique and by the immunofluorescence assay using the monoclonal antibody T11. Discrepancies were often observed with the immunofluorescence assay giving higher values.

Cytotoxic activities of the LGLs. Cytotoxicity assays showed that six out of six patients with LGLs of the T3+, T8+ phenotype had low or absent NK activity against K562 (Table 4). Cells from patients 4, 6, and 7 were tested against a number of other target cells and no NK activity could be demonstrated (Fig 2). The cells from patient 8 showed very strong ADCC activity using P815 as the target cell. Cells from most of the other patients tested exhibited only moderate to low levels of cytotoxicity toward P815. Cells from patients 1 and 3 with the T11+, T3−, T8−, M1+ phenotype, however, possessed powerful NK and ADCC activity.

A wide spectrum of cells were tested for their susceptibility to NK lysis by cells from patients 1 and 3. The three T cell lines CEM, MOLT-3, and KT were very sensitive, but HSB, RAJI, and all B lymphoblastoid cell lines tested were more

Table 4. Cytotoxic Activity of the Patients' LGL

		Patients							
E/T Ratio	Control	1	3	4	5	6	7	8	9
NK									
100	34(10)	108(2)	ND	4(2)	7(1)	3(3)	14(4)	14(1)	9(1)
50	28(11)	107(3)	81(1)	4(3)	5(1)	1(3)	8(4)	9(1)	5(1)
25	17(11)	92(3)	61(1)	1(3)	0(1)	0(3)	5(3)	6(1)	4(1)
10	24(3)	55(2)	57(1)	ND	ND	ND	2(1)	ND	1(1)
5	8(3)	25(2)	29(1)	ND	ND	ND	1(1)	ND	ND
ADCC									
100	71(2)	66(2)	ND	17(1)	ND	0(1)	12(1)	ND	ND
50	44(11)	58(2)	ND	14(2)	ND	2(2)	10(3)	120(1)	62(1)
25	25(10)	41(2)	90(1)	7(2)	ND	1(2)	6(3)	105(1)	29(1)
10	9(10)	9(2)	86(1)	3(2)	ND	0(2)	2(3)	62(1)	ND
5	0(2)	2(2)	84(1)	0(1)	ND	0(1)	ND	ND	ND

Abbrev: E/T ratio, effector to target cells ratio; ND, not done.

The values given are the percent cytotoxicity expressed to the nearest integer and represent the mean of the number of experiments indicated in parenthesis. NK assays were performed using K562 cells and ADCC using the P815 cells as targets.

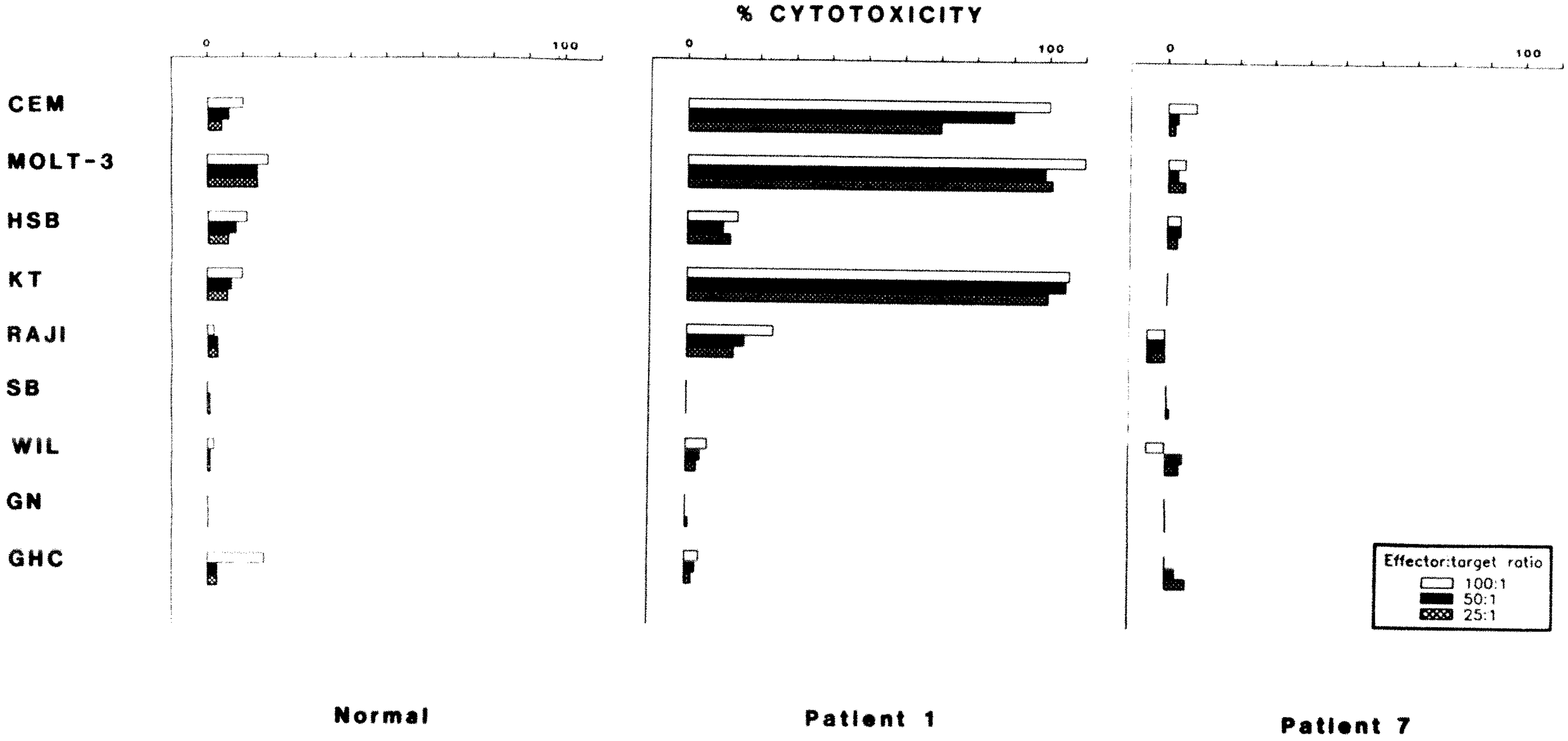

Fig 2. The spectrum of NK activity assessed using cell lines other than K562. Cells from patient 1 showed strong cytolytic function toward the 3 T-cell lines CEM, MOLT-3, and KT. They also had weak activity against HSB and RAJI but almost no activity against the B-lymphoblastoid lines SB, WIL, GN, and GHC. Cells from patient 3, another Type B patient, showed an identical pattern of cytolytic activity (data not shown). Cells from Type A patients, as illustrated in an experiment using cells from patient 7, demonstrated little activity against the target cells tested. Negative values indicate that the test was not performed.

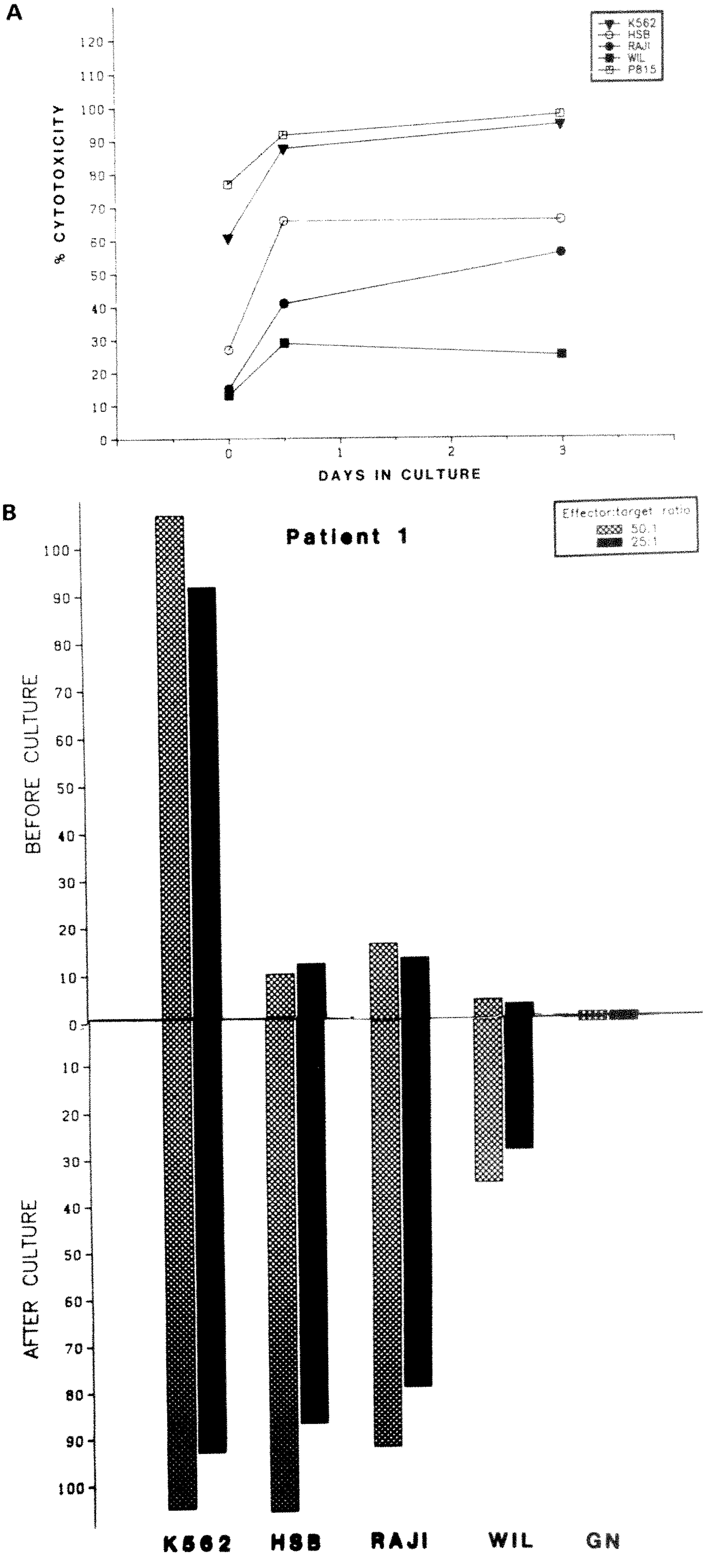

Fig 3. (A) NK and ADCC activity of the cells from patient 3 assayed when freshly isolated, at 12 hours, and three days after culture in RPM1 1640 with 10% fetal calf serum. Cytolytic function increased rapidly within 12 hours followed by slight or no further increase up to three days. The effector:target ratio was 25:1. (B) The changes in NK function of cells from patient 1 after five days of culture were similar to those observed in patient 3. Marked increase in lytic activity against HSB and RAJI was evident. There was also a modest activity against WIL but practically no NK function toward GN was observed.

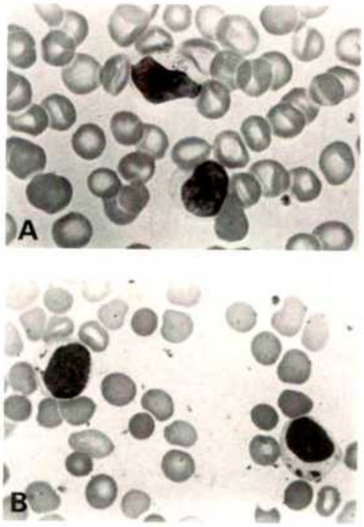

Fig 4. (A) The typical morphology of LGLs. The cytoplasmic granules are generally small and not conspicuous. (B) Cells from patient 8 showed exceptionally large cytoplasmic azurophilic granules (Wright Giemsa, original magnification ×1,000).

resistant (Fig 2). After culturing for 12 hours to five days, the cells from both patients acquired strong lytic activity against HSB, RAJI, and moderate activity against WIL but the other B lymphoblastoid cell lines (SB, GN, GHC) remained resistant. Cytolytic activity against K562 and antibody-coated P815 was also enhanced postculture (Figs 3A, B).

Morphology and cytochemistry of LGLs. Morphologically, the LGLs from patient 8 were very distinctive in that they contained very large prominent granules (Fig 4B). The vast majority of LGLs from the other patients contained fine azurophilic granules (Fig 4A). Only occasionally were larger granules seen. Ultrastructurally, the large granules consisted of parallel tubular arrays (PTA) (Fig 5A) similar in structure to those of the smaller granules in LGLs from patients 6 and 7. The LGLs from patient 1 contained both PTA and dense core granules (Fig 5B) whereas those from patient 3 contained almost exclusively dense core granules (Fig 5C). The LGLs from all patients exhibited ACP and BG activity. ANAE could not be demonstrated.

Response of type B LGL to IL-2 and TPA. The PBL from patient 1 proliferated vigorously in IL-2–containing medium and retained their basic T11+, T3−, T8−, T4−

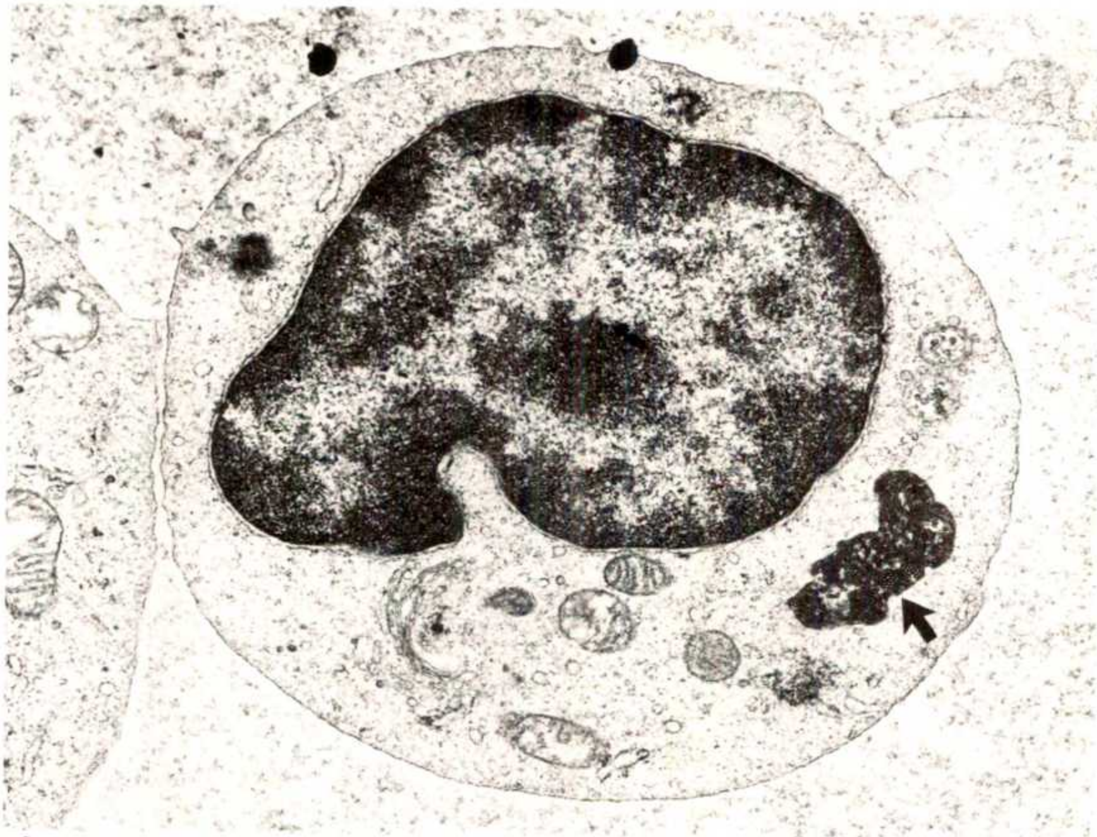

Fig 5. (A) LGL from patient 8 showing that the large azurophilic granules had the structure of parallel tubular arrays at the ultrastructural level (arrow). (B) LGL from patient 1 containing cytoplasmic structures with the morphology of parallel tubular arrays (arrow) and dense core granules (arrowhead). (C) LGL from patient 3 containing granules with an electron dense core surrounded by membrane (arrowhead). No parallel tubular arrays were observed. (Uranyl acetate and lead citrate, original magnification ×25,190, current magnification ×21,411.)

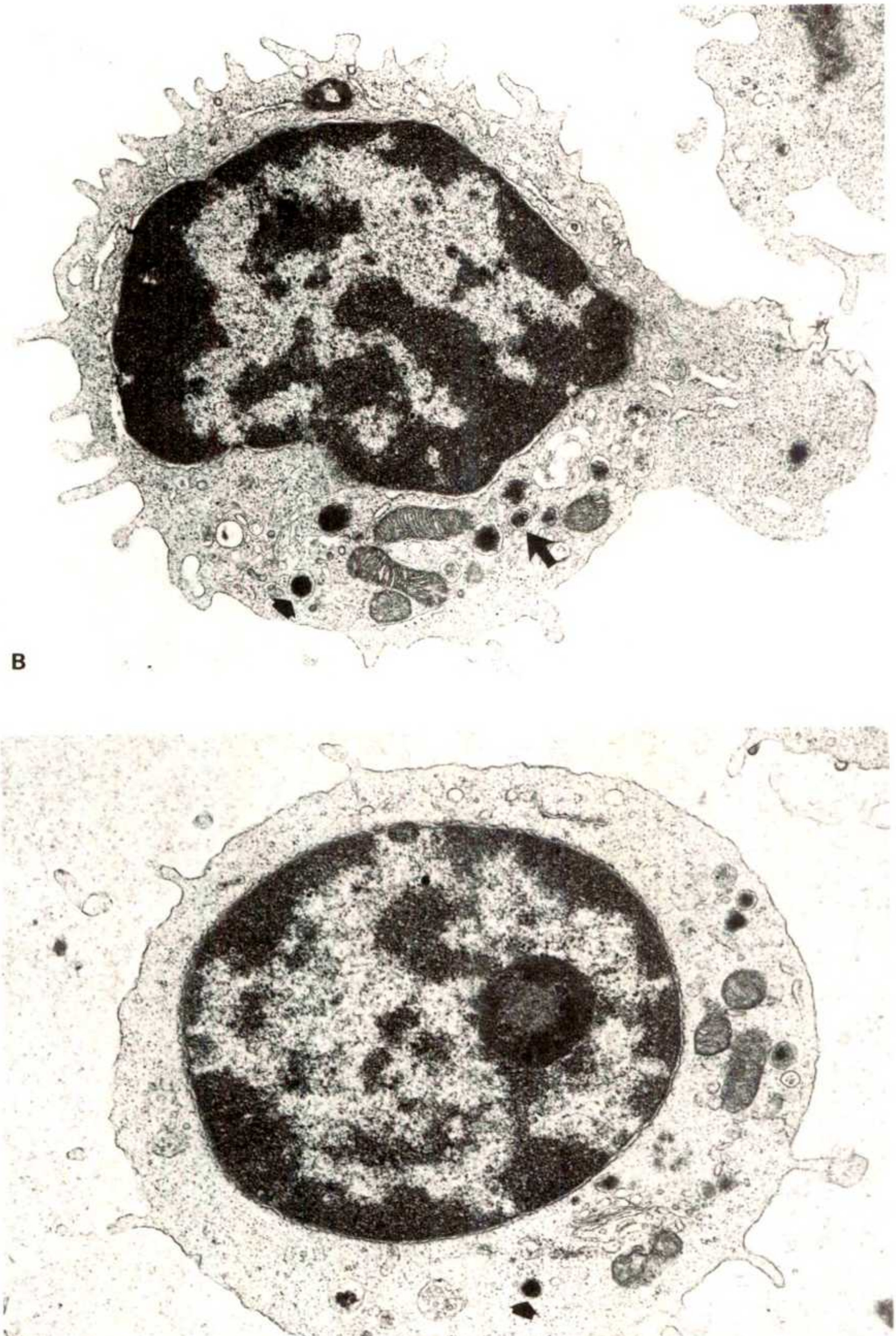

Fig 5. (Cont'd.)

phenotype. Strong NK activity also persisted (Fig 6). Cultured cells from patient 3 contained a substantial number of T3+, T8+ cells presumably derived from T3+ and T8+ cells present originally in the PBL. Repeat culture after eliminating T3+, T8+ cells from the PBL by complement-mediated lysis gave rise to cells with an almost identical phenotype to those of patient 1 (Fig 6). The cultured cells possessed very strong NK activity. PBL from both patients responded rapidly to TPA over a wide range of concentrations (Fig 7). The phenotype of the proliferating cells from patient 1 was similar to that of freshly isolated cells. A substantial number of T3+ cells was present in the culture of patient 2 probably resulting from proliferation of the residual normal T lymphocytes present as in the case of the IL-2 culture. Cytolytic activity from TPA-activated cells was very much diminished in both patients (Fig 8).

DISCUSSION

The 11 patients in this study may be divided into 2 major types. Type A showed a proliferation of LGL with a phenotype similar to that expressed by neonatal peripheral blood and normal adult bone marrow LGLs (T11+, T3+, T8+, T4−, M1−).[32] The majority of patients belonged to this group (9/11) and all suffered from neutropenia. Autoantibodies were frequently present and rheumatoid arthritis was clinically diagnosed in three. The LGLs showed variable ADCC activity and only cells from patients 8 and 9 showed substantial activity against P815. NK activity was weak or absent. The overwhelming majority of cases of LGL lymphocytosis reported in the literature have the Type A immunophenotype and clinicopathologic features. The case reported by Schlimok et al[12] had cells that also expressed the OKM1-defined antigen. That case is unusual since it was characterized by a very high white count, marked splenomegaly, and the peripheral blood cells had strong NK activity. Itoh et al[16] reported another unusual case that was T3+ but T8−. The cells acquired NK function after overnight culture.

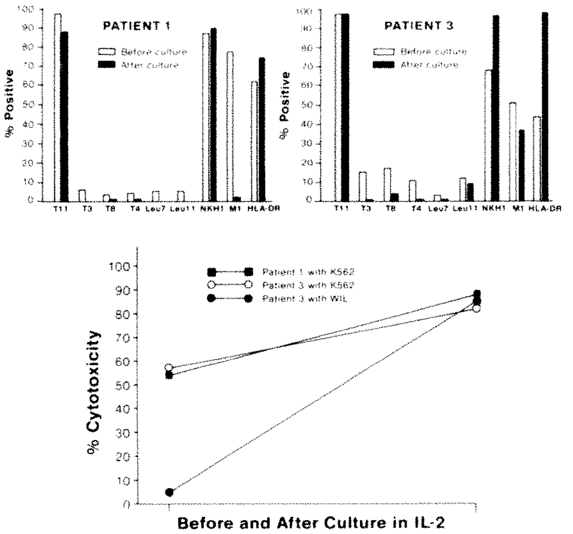

Fig 6. Surface markers and NK activity of Type B LGL after culture in IL-2–containing medium. PBL from patient 3 were incubated with T3 and T8 with complement to eliminate reactive cells before culture. The phenotype of the cells after culture (six and 11 days for patients 1 and 3, respectively) remained essentially unchanged except for the decrease in M1-bearing cells and further increase in cells bearing HLA-DR and NKH1 antigens. The cytolytic activity of the cultured cells was enhanced. This was especially well illustrated when WIL was used as the target. The effector:target ratio was 10:1.

The Type B phenotype (T11+, T3−, T8−, T4−, M1+) was less frequently encountered in the present series (2/11 patients). These two patients had not been followed as long as the other patients but no significant progression of their disease has been observed during the 8- and 5-month periods of observation. In contrast to the patients with Type A disease, they did not have neutropenia, autoantibodies, or hypergammaglobulinemia. The lack of clinical manifestations attributable to the lymphocytosis and the absence of other hematologic abnormalities may fail to bring the Type B patients to medical attention. This may account for the low incidence of this type of LGL lymphocytosis since only patients with substantial peripheral lymphocytosis or an unusually aggressive clinical course[19] would be detected. More Type B patients have to be studied, however, to determine whether the clinical features presented here are consistently observed in this type of LGL lymphocytosis.

The Type B LGLs possessed very strong ADCC and NK activity. In addition to K562, they were able to lyse the three T cell lines tested. Culturing the cells in medium with 10% FCS expanded their cytolytic spectrum. HSB and RAJI which were initially relatively resistant became highly susceptible, but the B lymphoblastoid cell lines were generally far less susceptible to lysis even when cultured effector cells were used. The increase in cytolytic activity was rapid with most of the changes occurring within the first 12 hours. The possibility of in vitro production by these cells of lymphokines such as the interferons, that enhance cytotoxic function, merits further investigation.

Among the Type A patients, heterogeneity in morphology, immunophenotype, and function were observed. The expression of T11, T3, and T8 was a consistent feature in the LGLs of this type of proliferation. The percentage of T11+ cells was always higher than the percentage of cells forming sheep red cell rosettes probably because of the disruption of some rosettes during the assay and the extent of disruption may depend on the density and avidity of the receptors. Leu 7, an antigen expressed by most normal LGLs, was present on a high proportion of cells in 4/5 Type A patients. LGLs from patient 4 did not express Leu 7, and were also unusual since they expressed M1, an antigen absent from all other Type A cases tested. The LGLs from patient 8 were morphologically distinctive since they contained very large granules. They also differed functionally from those of other patients by showing very strong ADCC activity against P815.

In normal peripheral blood, most LGLs react with anti-Leu 11, a monoclonal antibody against a type of Fc receptor present also on granulocytes.[30] NKH1[33] is another monoclonal antibody that reacts specifically with LGLs. Many but not all LGLs from normal subjects coexpress the antigens defined by these two antibodies. Cells from Type B patients, however, did not react with anti-Leu 11 but reacted well with

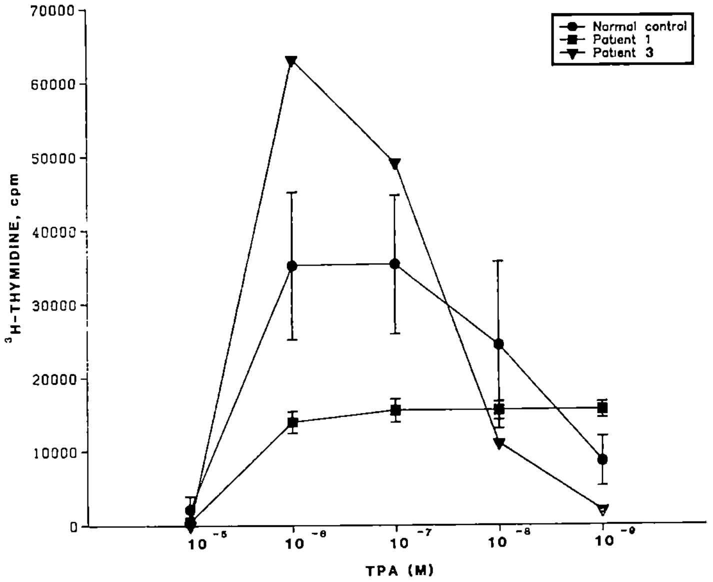

Fig 7. Tritiated thymidine uptake on the 3rd day after culture in various concentrations of TPA. Values for the normal control represent the mean of 3 experiments ± the standard error of the mean. Two experiments were performed on cells from patient 1 and one experiment on cells from patient 3. The concentration range of TPA effective in stimulating PBL from both normal individual and patient 1 was wide. It appeared to be narrower for PBL from patient 3.

NKH1, suggesting that they were derived from the Leu 11−, NKH1+ subpopulation. The Type A patients tested, however, generally failed to react with either antibodies. It should be noted that a high proportion of cells from these patients had Fc receptors demonstrable by EA rosette formation or other methodology both in the present series and in those reported in the literature.[5–7,9–12,16,17] It is possible that the EA rosette receptor and the Leu 11 antigen are two separable though closely associated entities. On the other hand, the Fc receptor on these LGLs may lack the Leu 11 epitope or the orientation of the molecule is such that the epitope is not available for binding with the antibody. Posttranslational modification of the epitope rendering it nonreactive with the antibody is another possible explanation.

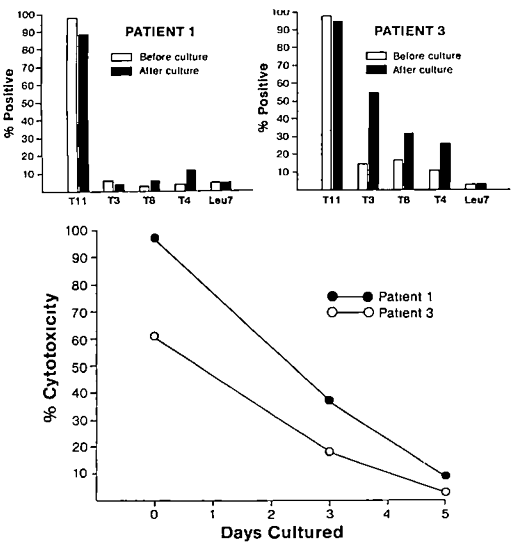

Fig 8. Surface markers and NK activity of Type B LGL after culture in medium containing 10^{-7} mol/L TPA. The expression of T11, T3, T8, T4, and Leu 7 was assayed on the 5th day of culture. They were essentially unchanged for patient 1 but a substantially higher percentage of T3+, T8+, and T4+ cells was seen in cultures of PBL from patient 3 presumably from proliferation of T3+ lymphocytes initially present in the PBL. There was a marked progressive decrease in the NK activity of the cultured cells. This difference could not be accounted for by a dilution effect due to the increase in T3+ cells from patient 3 since over 40% of T11+, T3− cells were still present. The effector:target ratio was 25:1.

The cytoplasmic granules of LGLs with different immunophenotypes contained the lysosomal enzymes acid phosphatase and beta-glucuronidase. Ultrastructurally, the granules of T3+, T8+ LGLs consisted almost exclusively of PTA with few dense core or lysosomal type granules. The granules from the LGLs of patient 3, a Type B patient, had the structure of dense core granules without PTA whereas the LGLs of patient 1 contained granules of both types.

It is interesting to note that in rat LGL tumors, the granules have been shown to contain cytolytic factor(s).[36] Contact of an LGL with a suitable target cell probably induces the release of granule contents producing membrane lesions similar to those caused by complement. It is highly probable that human LGL granules are functionally similar to rat LGL granules. Two types of granules, PTA and dense core granules, are, however, observed in human LGLs and their relationship is not clear. The LGLs with strong NK activity from patient 3 contained almost exclusively dense core granules. LGLs from patient 8 possessed very strong ADCC activity and those cells had granules with the structure of PTA. If the granules in human LGLs are also the structure with the cytolytic factor(s), then both PTA and dense core granules would appear to be in the category of cytolytic granules.

Bone marrow examination in both types of patients showed a variable degree of lymphocytic infiltration that did not always correlate with the peripheral LGL counts. Although Type A LGL lymphocytosis showed a remarkable association with neutropenia, the role of LGLs in its pathogenesis is still unclear. There were no uniform changes in the cellularity and maturation of the granulocytic series in these patients. The neutropenia accounted for most of the morbidity and one fatality observed, while the death of another patient might also be caused by fulminant septicemia. This aspect of the disease deserves further emphasis and research. Reticulin fibrosis was observed in all evaluable bone marrow biopsies but to varying degrees. The fibrosis may represent a reaction of the marrow to the lymphocytic infiltration, or alternatively it may be induced by a lymphokine(s) liberated by the lymphocytes.

The cell lineage of LGLs is not clear and may be heterogeneous. Nine of our 11 patients demonstrated an expansion of cells bearing a number of T cell markers including T11, T3, and T8. Four of the five Type A patients studied had cells showing clonal rearrangement of the gene for the beta chain of the T cell receptor.[37,38] Aisenberg et al[39] studied three patients with the same phenotype and also demonstrated clonal T-beta gene rearrangements in two. These findings offer additional strong evidence that LGLs with the T3+, T8+ phenotype belong to the T cell lineage. The monoclonal pattern of the T-beta chain gene rearrangement would also strongly imply a neoplastic rather than a reactive proliferation of the LGLs.[38] Patient 6, one of those with evidence of a clonal proliferation, however, had a spontaneous remission that has lasted for over 2 years.[21] From the generally indolent course of this disease and the occurrence of spontaneous remissions,[21,26] it is evident that in most cases the monoclonal proliferation behaves in a clinically benign fashion and may not be entirely autonomous but rather retain a degree of immunoregulatory control.

Type B cells expressed T11 but no other T cell antigens Since T3 is closely associated with the T cell receptor,[40] it may be the most specific marker for mature T lymphocytes. The lack of this surface antigen may indicate that the LGLs from Type B patients do not belong to the T cell lineage in contrast to cells from Type A patients. The T-beta gene also appeared to remain in the germ line state on Southern blot analysis after BamH1 and EcoR1 digestion in both of our Type B patients.[37] These results are similar to those recently reported by Rambaldi et al.[41]

Since the lineage of Type B LGLs is still unclear, attempts were made to stimulate the cells with IL-2 and TPA to observe changes that might define the origin of this type of cell. The LGLs from the two Type B patients proliferated well in both IL-2– and TPA-containing medium. The basic immunophenotype (T11+, T3−, T8−, T4−) of LGLs from patient 1 remained unchanged. PBL from patient 3 contained about 18% T3+ cells and, after a period of culture with either IL-2 or TPA, about half of the cells were T3+. T3+ and T8+ cells were eliminated by complement lysis before culture. Eleven days after IL-2 stimulation, the cultured cells had an almost identical phenotype to the cultured cells of patient 1. This suggests that there was no conversion of T3− cells to T3+ cells and that T3+ cells present after culture were derived from T3+ cells in the PBL, presumably normal residual T lymphocytes. The PBL of these patients might contain other populations of NK cells with the T11+, T3− phenotype aside from the expanded population under study and they all might have responded to IL-2 and TPA. Since this phenotype was maintained during culture, the conclusion of the lack of conversion to the T3+ phenotype, however, still holds. It has been suggested that NK cells with an immunophenotype similar to our Type B LGLs are a form of pre–T lymphocyte.[42] We have not been able to induce expression of T3 or other T cell markers on these cells. It is possible that these cells have undergone the initial phase of T cell development as indicated by the expression of T11, an antigen detected in early T cell ontogeny, but that they are unable to proceed further along the T cell differentiation pathway. Alternatively, these cells may belong to a separate lineage. Although both IL-2 and TPA induced proliferation of Type B LGLs, they had different influences on the NK function of the stimulated cells. Cells grown in IL-2 demonstrated enhanced cytolytic activity while those grown in TPA had markedly diminished NK function. The mechanism by which these agents alter NK cell function, whether directly or indirectly via monocytes or other cells in the same culture, is an interesting subject for future research.

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REFERENCES

1. Saksela E, Timonen T, Ranki A, Hayry P. Morphological and functional characterization of isolated effector cells responsible for human natural killer activity to fetal fibroblasts and to cultured cell line targets. Immunol Rev 44.71, 1979

2. Timonen T, Ortaldo JR, Herberman RB· Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153·569, 1981

3 Trinchieri G, Perussia B· Human natural killer cells. Biologic and pathologic aspects Lab Invest 50 489, 1984

4. McKenna RW, Parkin J, Kersey JH, Gail-Peczalska KJ, Peterson L, Brunning RD: Chronic lymphoproliferative disorder with unusual clinical, morphologic, ultrastructural and membrane surface marker characteristics. Am J Med 62:588, 1977

5 Bom-Van Noorloos AA, Pegels HG, Van Oers RHJ, et al: Proliferation of T-cells with killer-cell activity in two patients with neutropenia and recurrent infections. N Engl J Med 302:933, 1980

6 Callard RE, Smith CM, Worman C, Linch D, Crawley JC, Beverley PCL: Unusual phenotype and function of an expanded subpopulation of T cells in patients with haemopoietic disorders. Clin Exp Immunol 43:497, 1981

7. Linch DC, Cawley JC, Worman CP, Galvin MC, Roberts BE, Callard RE, Beverley PCL: Abnormalities of T-cell subsets in patients with neutropenia and an excess of lymphocytes in the bone marrow Br J Haematol 48 137, 1981

8 Aisenberg AC, Wilkes BM, Harris NL, Ault KA, Carey RW·

Chronic T-cell lymphocytosis with neutropenia: Report of a case studied with monoclonal antibody Blood 58·818, 1981

9. Nagasawa T, Abe T, Nakagawa T: Pure red cell aplasia and hypogammaglobulinemia associated with Tr-cell chronic lymphocytic leukemia. Blood 57:1025, 1981

10. Hooks JJ, Haynes BF, Detrick-Hooks B, Diehl LF, Gerrard TL, Fauci AS: Gamma (immune) interferon production by leukocytes from a patient with T_G cell proliferative disease. Blood 59:198, 1982

11 Pandolfi F, Rossi GD, Semenzato G, Quinti I, Rannucci A, De Sanctis G, Lopez M, Gasparotto G, Aiuti F: Immunologic evaluation of T-chronic lymphocyte leukemia cells: Correlations among phenotype, functional activities and morphology. Blood 59.688, 1982

12. Schlimok G, Thiel E, Rieber EP, Huhn D, Feucht H, Lohmeyer J, Riethmüller G: Chronic leukemia with a hybrid surface phenotype (T lymphocyte/myelomonocytic): Leukemic cells displaying natural killer activity and antibody-dependent cellular cytotoxicity. Blood 59·1157, 1982

13. Brisbane JU, Berman LD, Osband ME, Neiman RS: T8 chronic lymphocytic leukemia: A distinctive disorder related to T8 lymphocytosis. Am J Clin Pathol 80:391, 1983

14. Hocking WG, Singh R, Schroff R, Golde DW· Cell mediated inhibition of erythropoiesis and megaloblastic anemia in T-cell chronic lymphocytic leukemia. Cancer 51:631, 1983

15. Feller AC, Parwaresch MR, Lennert K: Subtyping of chronic lymphocytic leukemia of T-type by dipeptidylaminopeptidase IV (DAP IV), monoclonal antibodies and Fc-receptors. Cancer 52:1609, 1983

16. Itoh K, Tsuchikawa K, Awataguchi T, Shiba K, Kumagai K· A case of chronic lymphocytic leukemia with properties characteristic of natural killer cells. Blood 61:940, 1983

17. Palutke M, Eisenberg L, Kaplan, J, Hussain M, Kithier K, Tabaczka P, Mirchandani I, Tenebaum D: Natural killer and suppressor T-cell chronic lymphocytic leukemia. Blood 62:627, 1983

18. Phyliky RL, Li CY, Yam LT: T-cell chronic lymphocytic leukemia with morphologic and immunologic characteristics of cytotoxic/suppressor phenotype. Mayo Clin Proc 58.709, 1983

19. Tagawa S, Konishi I, Kuratune H, Katagiri S, Taniguchi N, Tamaki T, Inoue R, Kanayama Y, Tsubakio T, Machii T, Yonezawa T, Kitani T: A case of T-cell chronic lymphocytic leukemia (T-CLL) expressing a peculiar phenotype (E+, OKM1+, Leu1+, OKT3−, and IgG EA−). Cancer 52:1378, 1983

20 Bakri K, Ezdinli EZ, Wasser LP, et al: T-suppressor cell chronic lymphocytic leukemia· Phenotypic characterization by monoclonal antibodies. Cancer 54:284, 1984

21. Chan WC, Winton EF, Check IJ: T Cell imbalance in neutropenia of uncertain etiology. Am J Clin Pathol 81:54, 1984

22. Chan WC, Check I, Schick C, Brynes RK, Kateley J, Winton EF: A morphologic and immunologic study of the large granular lymphocyte in neutropenia with T-lymphocytosis. Blood 63·1133, 1984

23. Newland AC, Catovsky D, Linch D, Cawley JC, Beverley P, San Miguel JF, Gordon-Smith EC, Blecher TE, Shahriari S, Varadi S: Chronic T cell lymphocytosis. A review of 21 cases. Brit J Haematol 58 433, 1984

24. Semenzato G, Pizzolo G, Ranucci A, Agostini M, Chilosi I, Quinti G, De Sanctis B, Vercelli B, Pandolfi F: Abnormal expansion of polyclonal large to small size granular lymphocytes: Reactive or neoplastic process? Blood 63:1271, 1984

25. Loughran TP, Kadin ME, Starkebaum G, Abkowitz JL, Clark EA, Disteche C, Lum LG, Slichter SJ: Leukemia of large granular lymphocytes· Association with clonal chromosomal abnormalities and autoimmune neutropenia, thrombocytopenia and hemolytic anemia Ann Int Med 102:169, 1985

26 McKenna RW, Arthur DC, GajL-Peczalska NK, Flynn P, Brunning RD. Granulated T-cell lymphocytosis with neutropenia: Malignant or benign chronic lymphoproliferative disorder? Blood 66 259, 1985

27 Luna LG: Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology (ed 3). New York, McGraw Hill, 1968, p 90

28. Lorbacher P, Yam LT, Mitus WJ· Cytochemical demonstration of beta-glucuronidase activity in blood and bone marrow cells J Histochem Cytochem 15:680, 1967

29. Kamoun M, Martin PJ, Hansen JA, Brown MA, Siadek AW, Nowinski RC· Identification of human T-lymphocyte surface protein associated with the E-rosette receptors J Exp Med 153.207, 1981

30. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 131:1789, 1983

31. Reinherz EL, Schlossman SF: The differentiation and function of human T-lymphocytes. Cell 19:821, 1980

32. Abo T, Miller CA, Gartland GL, Balch CM: Differentiation stages of human natural killer cells in lymphoid tissue from fetal to adult life. J Exp Med 157:273, 1983

33 Hercend T, Griffin JD, Bensussan A, Schmidt RE, Edson MA, Brennan A, Murray C, Daley JF, Schlossman SF, Ritz J: Generation of monoclonal antibodies to a human natural killer clone. Characterization of two natural killer associated antigens, NKH1a and NKH2, expresses on subsets of large granular lymphocytes. J Clin Invest 75:932, 1985

34. Nicholson JKA, McDougal JS, Spira TJ, Cross GD, Jones BM, Reinherz EL: Immunoregulatory subsets of T-helper and T-suppressor cell populations in homosexual men with chronic unexplained lymphadenopathy J Clin Invest 73·191, 1984

35. Bolhuis RLH, Schuit HRE, Nooyen AM, Ronteltap CPM: Characterization of natural killer (NK) cells and killer (K) cells in human blood: Discrimination between NK and K cell activities Eur J Immunol 8:731, 1978

36. Henkart PA, Millard PJ, Reynolds CW, Henkart MP: Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. J Exp Med 180 75, 1984

37. Waldmann TA: Personal communication

38. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ: Rearrangements of genes for the antigen receptor on T-cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 313:776, 1985

39. Aisenberg AC, Krontiris TG, Mark TW, Wilkes BM Rearrangement of the gene for the beta chain of the T-cell receptor in T-cell chronic lymphocytic leukemia and related disorder. N Engl J Med 313 529, 1985

40. Reinherz EL, Acuto O, Fabbi M, Benussan A, Milanese C, Royer HD, Meuer SC, Schlossman SF: Clonotypic surface structure on human T lymphocytes: Functional and biochemical analysis of antigen receptor complex Immunol Rev 81:95, 1984

41. Rambaldi A, Pelicci PG, Allavena P, Knowles II DM, Rossini S, Bassan R, Barbui T, Dalla-Favera R, Montovani A. T cell receptor beta chain gene rearrangements in lymphoproliferative disorders of large granular lymphocytes/natural killer cells. J Exp Med 612:2156, 1985

42. Ritz J, Campen TJ, Schmidt RE, Royer HD, Hercend T, Hussey RE, Reinherz EL: Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones Science 228·1540, 1985

Regulation of Human Monocyte Surface Antigen Expression. I. Up-Modulation of Mo3e Antigen Expression on U-937 and HL-60 Cells Stimulated by Pharmacologic Activators of Protein Kinase C

By Robert F. Todd III, Martin J. Bury, Patti A. Alvarez, David A. Brott, and David Y. Liu

Mo3e is a protein (p 50,80) that is expressed on the surface of human monocytic cells after exposure in vitro to soluble activating factors that include bacterial lipopolysaccharide, muramyl dipeptide, and phorbol myristate acetate (PMA). The surface expression of Mo3e may represent a cellular event that occurs in response to the formation of "secondary messengers" that include diacylglycerol, inositol trisphosphate, and calcium ions. This postulate is based on the stimulatory effect of agents that can mimic the activity of endogenous diacylglycerol (PMA and other biologically active phorbol compounds, mezerein, and L-α-1,2 dioctanoylglycerol) and inositol trisphosphate (ionomycin) on Mo3e expression by U-937 and HL-60 cells. The inhibitory effect of phospholipid-active calmodulin inhibitors (trifluoperazine, chlorpromazine, and dibucaine), calcium antagonists (nicardipine and TMB-8), and EGTA further support the involvement of phospholipid- and calcium-dependent protein kinase (protein kinase C) and calcium ions in the up-modulation of Mo3e surface expression.

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WE HAVE RECENTLY REPORTED the identification of a plasma membrane antigen, Mo3e, that is expressed by human peripheral blood monocytes activated in culture by exposure to bacterial lipopolysaccharide, muramyl dipeptide, or biologically active phorbol compounds.[1] Mo3e, which consists of two polypeptides of 50 and 80 kd on Western blot analysis,[2] may represent the surface receptor for migration inhibitory factor (MIF), as evidenced by inhibition of monocyte MIF responsiveness produced by anti-Mo3e monoclonal antibody.[3]

In this report, we extend these initial observations with a consideration of the signal transduction pathways that may regulate the up-modulated expression of Mo3e on the surface of human mononuclear phagocytes. By immunofluorescence analysis, we demonstrate the up-modulated expression of Mo3e on the surface of human myeloid cell lines U-937 and HL-60 that have been exposed to agents that mimic the activity of endogenous diacylglycerol (phorbol myristate acetate [PMA] and other biologically active phorbol compounds, mezerein, and L-α-1,2 dioctanoylglycerol) or inositol trisphosphate (ionomycin), "second messengers" in the signal transduction pathway that involves the activation of phospholipid- and calcium-dependent protein kinase (protein kinase C) (see reference 4 for review). The involvement of this pathway is further supported by the inhibitory effect of phospholipid-active calmodulin inhibitors (trifluoperazine, chlorpromazine, and dibucaine[5,6]), EGTA, and intracellular calcium antagonists (nicardipine and TMB-8[7,8]) on PMA-stimulated Mo3e expression.

MATERIALS AND METHODS

Media and reagents The following media and tissue culture additives were purchased from GIBCO Laboratories (Grand Island, NY). RPMI 1640 (320-1875), L-glutamine 200 mmol/L (320-5030), and penicillin-streptomycin solution (600-5070). Fetal calf serum (FCS) was purchased from HyClone Laboratories (Logan, Utah). Lots of RPMI 1640 and FCS were preselected for low endotoxin activity (<0.1 ng/mL) The following reagents were purchased from Sigma Chemical Company (St Louis): 4β-phorbol (P; P8893); 4β-phorbol 12,13-dibutyrate (PDB, P1269); 4β-phorbol 12,13-didecanoate (βPDD; P9018); 4α-phorbol 12,13-didecanoate (αPDD; P8014); 4β-phorbol 12-myristate 13-acetate (PMA; P8139); dimethyl sulfoxide (DMSO; D5879); trifluoperazine dihydrochloride (T8516); chlorpromazine hydrochloride (C8138), dibucaine hydrochloride (D0638); nicardipine hydrochloride (N7510); 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester hydrochloride (TMB-8; T0517), ethylene glycolbis (β-amino-ethyl ether) *N, N', N'*-tetra-acetic acid (EGTA, E4378); and trans retinoic acid (R2625) Mezerein (M4600) was purchased from LC Services Corp (Woburn, Mass). L-α-1,2 Dioctanoylglycerol (800800) and L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol (800100) were purchased from Avanti Polar Lipids, Inc (Birmingham, Ala). Ionomycin from *Streptomycin conglobatus* (407952) was purchased from CalBiochem (San Diego) 1,25-Dihydroxycholecalciferol (Ro21-5535) was a gift of Dr M.R Uskokovic (Hoffman-La Roche Inc, Nutley, NJ) The phorbol compounds, mezerein, TMB-8, and ionomycin were dissolved in dimethyl sulfoxide (DMSO) and stored as a stock solution at −80 °C Stock solutions of nicardipine, 1,25-dihydroxycholecalciferol, and trans retinoic acid were prepared in 100% ethanol and stored at −80 °C. Just before use, working solutions of L-α-1,2 dioctanoylglycerol or L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol were prepared by evaporating the chloroform diluent under N_2 and dissolving the residue in 100% ethanol.

Monoclonal antibodies. The generation and characterization of monoclonal antibodies anti-Mo1 (IgM),[9] anti-Mo2 (IgM),[9] anti-Mo3e (IgM),[1] anti-Plt 1 (IgM),[10] anti-J13 (IgM),[11] anti-Mb1 (IgG1),[12] and anti-β_2M (IgG2b)[13] have been described. Anti-J13 and β_2M were gifts of Drs Jerome Ritz and Lee Nadler (Dana-Farber Cancer Institute, Boston) Antibody-containing ascites were used in all immunofluorescence assays

Human cell lines. The human histiocytic lymphoma cell line U-937[14] and the promyelocytic leukemia cell line HL-60[15] were obtained from Dr Thomas Huard (Simpson Memorial Research Institute, Ann Arbor, Mich) and grown in RPMI 1640-FCS (RPMI 1640 medium supplemented with 10% FCS, L-glutamine 2 mmol/L, penicillin 50 U/mL, and streptomycin 50 μg/mL). The cell lines were maintained in log growth phase by thrice-weekly feeding.

Culture of cell lines with various reagents. In experiments designed to assess the effect of various reagents on the expression of

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Mo3e by cultured U-937 and HL-60 cells, 3 to 4 × 10^5 cells were incubated in 100-mm plastic culture dishes (Falcon Plastics, Oxnard, Calif) containing fresh 1640-FCS to which various chemical agents were added (to a total volume of 10 mL). The final concentration of DMSO or ethanol, which was used as an initial diluent for certain reagents as indicated earlier, did not exceed 0.4% and cultures containing appropriate quantities of DMSO or ethanol were run in parallel to control for any minor diluent effects. In certain experiments, U-937 cells were preincubated for one hour at 37 °C in 1640-FCS containing PMA, washed three times, and incubated in 10 mL of PMA-free 1640-FCS for various durations. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 22 to 120 hours. For culture durations exceeding 48 hours, 5 mL of fresh 1640-FCS was added to each culture dish. At the end of culture, medium containing nonadherent cells were removed and combined with adherent cells harvested by exposure to 0.5 mmol/L EDTA followed by scraping.[1] These recovered nonadherent and adherent cells were washed, counted (viability assessed by trypan blue exclusion), and resuspended in wash medium (phosphate-buffered saline supplemented with glucose 1 mg/mL and 2.5% human pooled AB serum) at a concentration of 5 to 20 × 10^6 cells per milliliter for subsequent immunofluorescence staining. In experiments in which U-937 cells were cultured for 24 to 48 hours in the presence of various chemical additives, cell viability generally exceeded 80%. U-937 and HL-60 cells cultured for 72 to 120 hours in medium containing PMA at a concentration of >2 nmol/L (HL-60) or 5 nmol/L (U-937) experienced a gradual decline in viability that reached 40% to 50% by 96 to 120 hours.

Immunofluorescence flow cytometric analysis. Aliquots (100 μL) containing 0.5 to 2.0 × 10^6 cells were subjected to indirect immunofluorescence staining for the expression of Mo3e (and other cell surface markers) relative to background staining using an IgM-negative control reagent (anti-J13 or Plt 1), as described.[1] Intact (live) cells were selected by gating (log forward angle *v* log 90° light scatter). Immunofluorescence intensity as a measure of relative antigen expression was analyzed on a Coulter Electronics (Hialeah, Fla) EPICS C flow cytometer with the use of a logarithmic amplifier. The channel number (log scale) representing the mean fluorescence intensity (major fluorescence peak) of 5,000 cells exposed to either experimental or isotype-identical negative control antibodies was determined. The corresponding linear fluorescence intensity channel was calculated from a logarithmic-linear calibration formula.[16] Specific fluorescence intensity represents the computed mean channel number (0 through 296 channels, linear scale) of cells stained with the experimental monoclonal antibody minus the mean channel of cells stained with the negative control reagent. To enhance the comparison in specific fluorescence intensity between unstimulated and stimulated cells, individual experiments were often performed by using different fluorescence gains (as determined by the photomultiplier tube [PMT] setting), and the gain in millivolts is shown where indicated (fluorescence gain *within* a given experiment was held constant). For each experiment, the mean fluorescence intensity (linear channel number) of cells stained with the negative control antibody is indicated.

RESULTS

PMA stimulates a dose- and time-dependent increase in U-937 Mo3e expression. Exposure of U-937 cells to PMA stimulates an up-modulation in the surface expression of Mo3e.[1] As shown in Table 1, the magnitude of Mo3e expression depends on the concentration of PMA to which the cells are exposed. Either continuous culture of U-937 cells in medium containing 5 to 10 nmol/L PMA (experiments 1 and 2) or pulse (one hour) exposure of cells to 50 to 100 nmol/L PMA (experiments 3 and 4) results in maximal Mo3e surface density after 24 hours of incubation. The surface expression of plasma membrane antigens Mb1[12] and β_2M is relatively unaltered by this treatment, indicating the specificity of Mo3e up-modulation in response to PMA. Culture of U-937 cells for up to 120 hours either in the continuous presence of PMA (Table 2, experiments 1 and 2) or after pulse PMA exposure (experiment 3) stimulates a further increase in surface Mo3e expression, which is accompanied by a loss of proliferative activity (data not shown).

PMA stimulates the expression of Mo3e by HL-60 cells coincident with monocytic differentiation. We[10,17] and others[18-20] have previously shown that culture of HL-60 promyelocytic cells in medium containing PMA results in their differentiation into monocytic cells that acquire the expression of several myelomonocytic surface antigens. As shown in Table 3, these PMA-cultured cells also express Mo3e after 72 to 96 hours of incubation. However, exposure of HL-60 cells to 1,25-dihydroxycholecalciferol (D_3), which also induces monocytic differentiation[21,22] (as evidenced by the appearance of intense nonspecific esterase activity [not shown] and the acquired expression of antigens Mo1 and

Table 1. Mo3e Expression Stimulated by Continuous or Pulse Exposure of U-937 Cells to Various Concentrations of PMA

	Continuous PMA Exposure				60-Minute Pulse PMA Exposure			
PMA (nmol/L)	Experiment 1		Experiment 2	PMA (nmol/L)	Experiment 3		Experiment 4	
	Mo3e	Mb1	Mo3e		Mo3e	Mb1	Mo3e	β_2M
10	66.47 (0.37)	26.67	44.05 (0.36)	100	—		57.36 (0.40)	68.42
5	48.12 (0.36)	26.68	41.52 (0.37)	50	47.74 (0.74)	21.95	46.63 (0.45)	76.90
2.5	40.33 (0.36)	25.14	36.90 (0.38)	10	28.03 (0.63)	16.82	27.39 (0.45)	83.98
1.25	16.58 (0.36)	34.80	31.85 (0.36)	5	21.43 (0.61)	21.43	18.03 (0.47)	89.04
0.62	7.35 (0.35)	32.82	14.29 (0.35)	1	5.31 (0.61)	19.58	—	—
0.31	3.59 (0.35)	29.16	4.10 (0.32)	0	2.05 (0.49)	19.70	1.19 (0.40)	65.64
0	2.12 (0.35)	26.69	1.04 (0.34)					

U-937 cells were cultured (3 to 4 × 10^5 cells per milliliter) for 24 hours in 1640-FCS containing the indicated concentrations of PMA (experiments 1 and 2) or after a 60-minute pulse exposure to the indicated concentrations of PMA followed by washing (experiments 3 and 4). After culture, the cells were stained by indirect immunofluorescence for the expression of Mo3e, Mb1, or β_2M. In a separate experiment, Mo3e expression by isolated adherent and nonadherent fractions (see Materials and Methods) was nearly identical (not shown). PMT settings for experiments 1, 2, 3, and 4 were 1,050, 1,000, 1,000, and 1,000 mV, respectively. Numbers indicate specific fluorescence intensity as calculated in Materials and Methods. Numbers in parentheses indicate fluorescence intensity of cells stained with a negative control antibody

Table 2. Mo3e Expression Stimulated by Exposure of U-937 Cells to PMA as a Function of Culture Duration

Experiment	PMA	Duration of Culture (hours) 24	48	72	96	120
1	4 nmol/L	16.62 (0.32)	—	50.87 (0.52)	—	70.02 (0.84)
	0	1.21 (0.34)	—	1.00 (0.30)	—	0.47 (0.32)
2	5 nmol/L	21.07 (0.33)	—	63.33 (0.49)	—	129.53 (1.30)
	0	0.40 (0.30)	—	0.29 (0.25)	—	0.14 (0.30)
3	50 nmol/L	58.77 (0.70)	87.72 (1.79)	203.00 (5.75)	230.30 (11.26)	—
	0	2.99 (0.61)	2.50 (0.44)	2.12 (0.49)	2.70 (0.51)	—

U-937 cells were cultured (3 to 4 × 10^6 cells per milliliter) for 24 to 120 hours in 1640-FCS containing the indicated concentrations of PMA (or DMSO diluent [0]) (experiments 1 and 2); in experiment 3, U-937 cells were exposed to PMA (or DMSO diluent) for 60 minutes at 37 °C, washed, and cultured in PMA-free medium. At the end of incubation, the cells were stained by indirect immunofluorescence for the expression of Mo3e. In a separate experiment, Mo3e expression by isolated adherent and nonadherent fractions was nearly identical (not shown). PMT settings in experiments 1, 2, and 3 were 1,000, 950, and 1,050 mV, respectively. Numbers indicate the specific fluorescence intensity as calculated in Materials and Methods. Numbers in parentheses indicate fluorescence intensity of cells stained with a negative control antibody

Mo2 [Table 3]), and to agents that stimulate granulocytic differentiation (DMSO, 1.2%, and retinoic acid, 1 μmol/L[23,24]) (data not shown) does not result in significant Mo3e expression (Table 3). Monocytic differentiation of HL-60 cells stimulated by 72 hours' exposure to D_3 does, however, "prime" these Mo3e-negative cells to respond more rapidly to a subsequent PMA stimulus with the appearance of Mo3e after only 24 hours of culture (Table 4). These observations demonstrate that the acquired expression of Mo3e is not an invariant feature of monocytic differentiation per se, but rather its appearance in this HL-60 line is a selective response to PMA.*

Association between Mo3e up-modulation and protein kinase C activation. Mo3e surface expression on monocytes and U-937 cells is stimulated not only by PMA, but also by other compounds that activate calcium-and phospholipid-dependent protein kinase (protein kinase C).[4] As shown in Table 5, 24-hour culture of U-937 cells in medium containing 5 nmol/L PMA, PDB, βPDD, or mezerein results in a reproducible up-modulation of Mo3e surface expression (the biologically inert compounds αPDD and P are inactive). These observations suggest an association between stimulation of Mo3e up-modulation and protein kinase C activation. This notion is further supported by the fact that exposure of U-937 cells to a diacylglycerol compound (the endogenous activator of protein kinase C[4]) likewise results in a reproducible increase in Mo3e surface density. As indicated in Table 5, repetitive doses of L-α-1,2 dioctanoylglycerol (final concentration of 0.13 to 0.14 mmol/L of this rapidly metabolized compound[27,28]) induces a fourfold to eightfold increase in Mo3e surface expression after 24 hours of culture relative to parallel diluent (ethanol)-treated control cells. Exposure of U-937 cells to another cell-permeable diacylglycerol compound, L-α-oleoyl-2-acetoyl-*sn*-3-glycerol, also induces an up-modulation in surface Mo3e expression (not shown). On the basis of these findings, we postulate that the up-modulated expression of Mo3e may be among the cellular responses initiated by protein kinase C activation.

Modulation of PMA-induced Mo3e surface expression by agents that influence calcium metabolism. Intracellular calcium is required as a "second messenger" in the initiation of certain biological responses stimulated by protein kinase C activation.[4] To determine whether Mo3e up-modulation is among these calcium-dependent kinase-stimulated responses, we assessed the effect of the calcium ionophore ionomycin on Mo3e expression stimulated by PMA. As shown in Fig 1, ionomycin (which may mimic the effect of endogenous inositol 1,4,5- trisphosphate in mobilizing calcium ions from intracellular stores[4,29,30]) has a synergistic effect on Mo3e expression stimulated by PMA. In a series of five experiments, the addition of ionomycin 2 μmol/L to cultures containing PMA 0.5 to 1.0 nmol/L resulted in a 3.1 (±1.1)- to 1.8 (±0.5)-fold increase, respectively, in Mo3e expression above that seen in cells exposed to PMA alone.

The requirement for calcium ions in the regulation of surface Mo3e expression is also suggested by the inhibitory effect of several "calcium antagonists." As indicated in Table 6, PMA-stimulated Mo3e expression is blocked by the inclusion of trifluoperazine, chlorpromazine, or dibucaine. This class of compounds is known to inhibit calmodulin-dependent responses[31,32] but may also block calmodulin-independent protein kinase C activation.[5,6] Mo3e up-modulation is also inhibited by nicardipine and TMB-8, agents that block intracellular mobilization and calcium influx into mammalian cells [7,8,33] A requirement for extracellular calcium for PMA-stimulated Mo3e expression is supported by the inhibitory effect of EGTA (which is reversed by the addition of calcium, but not magnesium, ions).

These observations taken together suggest the importance of both extracellular and intracellular calcium in the promotion of Mo3e up-modulation stimulated by PMA.

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*The possibility that protein kinase C activation may be involved in monocytic differentiation of HL-60 cells stimulated by 1,25-dihydroxycholecalciferol (D_3) has, however, been suggested by Matsui et al, who reported a partial inhibition of HL-60 differentiation by a selective inhibitor of protein kinase C.[25] In examining several sublines of both HL-60 and U-937 (from different sources) for Mo3e expression after D_3 exposure, we have observed considerable heterogeneity, ranging from little or no stimulation (eg, the HL-60 line used in this report [Table 3 and 4]) to Mo3e expression comparable to that stimulated by PMA (a consistent inducer of Mo3e expression in all sublines tested) (R.F.T. III, unpublished observations, January 1986). This heterogeneity in response to D_3 among distinct sublines is consistent with the findings of others.[26]

Table 3. Mo3e Expression Stimulated by Exposure of HL-60 Cells to PMA but not to 1,25-Dihydroxycholecalciferol (D_3)

		72 h			96 h		
Experiment	Additive	Mo1	Mo2	Mo3e	Mo1	Mo2	Mo3e
1	PMA (4 nmol/L)	12.07	1.76	71.22 (1.74)	10.11	1.76	68.32 (2.54)
	0	0.37	0.15	0.34 (0.66)	0.09	0.04	0.04 (0.40)
	D_3 (0.1 µmol/L)	1.84	51.73	0.00 (0.19)	3.23	60.65	0.00 (0.59)
	0	0.36	0.00	0.08 (0.64)	0.09	0.33	0.06 (0.39)
2	PMA (4 nmol/L)	—	—	—	2.51	—	55.40 (0.70)
	0	—	—	—	0.10	—	0.53 (0.47)
	D_3 (0.1 µmol/L)	—	—	—	15.05	—	0.23 (0.47)
	0	—	—	—	0.14	—	0.28 (0.49)
3	PMA (4 nmol/L)	1.64	0.12	20.91 (0.49)	3.82	0.00	19.66 (3.03)
	0	0.00	0.00	0.02 (0.33)	0.02	0.00	0.02 (0.61)
	D_3 (0.5 µmol/L)	0.45	2.29	0.04 (0.32)	1.77	8.55	0.11 (0.63)
	0	0.05	0.05	0.05 (0.29)	0.05	0.00	0.00 (0.79)
4	PMA (4 nmol/L)	2.24	0.00	40.08 (0.61)	—	—	—
	0	0.02	0.01	0.05 (0.31)	—	—	—
	D_3 (0.5 µmol/L)	1.59	4.52	0.11 (0.31)	—	—	—
	0	0.02	0.02	0.05 (0.30)	—	—	—

HL-60 cells were cultured (3 to 4 × 10^5 cells per milliliter) for 72 to 96 hours in 1640-FCS containing the indicated concentrations of PMA (or DMSO diluent [0]) or 1,25-dihydroxycholecalciferol (D_3) (or ethanol diluent [0]). After culture, the cells were stained by indirect immunofluorescence for the expression of Mo1, Mo2, and Mo3e PMT setting in experiments 1, 3, and 4 were 1,100 mV; in experiment 2, 1,200 mV. Numbers indicate specific fluorescence intensity as calculated in Materials and Methods. Numbers in parentheses indicate fluorescence intensity of cells stained with a negative control antibody.

Table 4. Expression of Mo3e Stimulated by Exposure of D_3-Differentiated HL-60 Cells to PMA

D_3 (×72 h)	PMA (×24 h)	Experiment 1			Experiment 2			Experiment 3		
		Mo1	Mo2	Mo3e	Mo1	Mo2	Mo3e	Mo1	Mo2	Mo3e
0.5 µmol/L	4 nmol/L	3.21	13.20	11.68 (0.61)	3.96	45.37	9.09 (0.36)	2.52	29.00	9.80 (0.51)
0	4 nmol/L	0.11	0.09	2.69 (0.52)	0.06	0.07	0.90 (0.32)	0.08	0.03	1.07 (0.39)
0.5 µmol/L	0	1.67	5.87	0.13 (0.59)	4.25	57.46	0.12 (0.30)	4.92	52.41	1.08 (0.51)
0	0	0.10	0.16	0.23 (0.54)	0.03	0.07	0.00 (0.32)	0.06	0.02	0.00 (0.42)

HL-60 cells were cultured (3 × 10^5 cells per milliliter) for 72 hours in 1640-FCS with the indicated final concentrations of 1,25-dihydroxycholecalciferol (D_3) (or ethanol control [0]). PMA (or DMSO control [0]) was then added and the incubation continued for an additional 24 hours. After culture, the cells were stained by indirect immunofluorescence for the expression of Mo1, Mo2, or Mo3e. PMT setting in all three experiments was 1,100 mV. Numbers indicate the specific fluorescence intensity as calculated in Materials and Methods. Numbers in parentheses indicate fluorescence intensity of cells stained with a negative control antibody.

Table 5. Expression of Mo3e Stimulated by Exposure of U-937 Cells to Diacylglycerol (DG) and Other Agents That Activate Protein Kinase C

	Specific Fluorescence Intensity (Mo3e)			
Additive	Experiment 1	Experiment 2	Experiment 3	Experiment 4
DG (0.13 mmol/L)	7.39 (0.54)	5.45 (0.30)	13.73 (0.49)	19.17 (0.44)
Ethanol (0.26%)	0.88 (0.31)	1.27 (0.32)	2.21 (0.48)	3.32 (0.39)
PMA (5 nmol/L)	44.02 (0.39)	33.82 (0.33)	53.86 (0.63)	32.40 (0.77)
PDB (5 nmol/L)	3.50 (0.32)	7.18 (0.30)	9.50 (0.52)	—
βPDD (5 nmol/L)	25.15 (0.35)	21.07 (0.33)	42.55 (0.59)	—
Mezerein (5 nmol/L)	16.60 (0.34)	23.05 (0.31)	26.52 (0.52)	—
αPDD (5 nmol/L)	0.85 (0.34)	1.34 (0.30)	2.26 (0.51)	—
P (5 nmol/L)	1.12 (0.34)	1.44 (0.30)	1.72 (0.54)	—
DMSO (0.00034%)	0.75 (0.34)	1.77 (0.30)	1.93 (0.54)	1.75 (0.44)

U-937 cells were cultured (3 × 10^5 cells per milliliter) for 24 hours in 1640-FCS containing the indicated final concentrations of additives DG (L-α-1,2 dioctanolglycerol) was added as a loading dose (50 µmol/L), followed by eight to nine hourly doses of 10 µmol/L (ethanol diluent was added to control cultures in parallel). In experiment 3, the final DG concentration was 0.14 mmol/L (final ethanol concentration was 0.28%) In a separate experiment, Mo3e expression by isolated adherent and nonadherent fractions after DG exposure was nearly identical (not shown) PMT setting in experiments 1 and 4 were 1,100 mV; in experiments 2 and 3, 1,150 mV. Numbers indicate the specific fluorescence intensity as calculated in Materials and Methods. Numbers in parentheses indicate fluorescence intensity of cells stained with a negative control antibody.

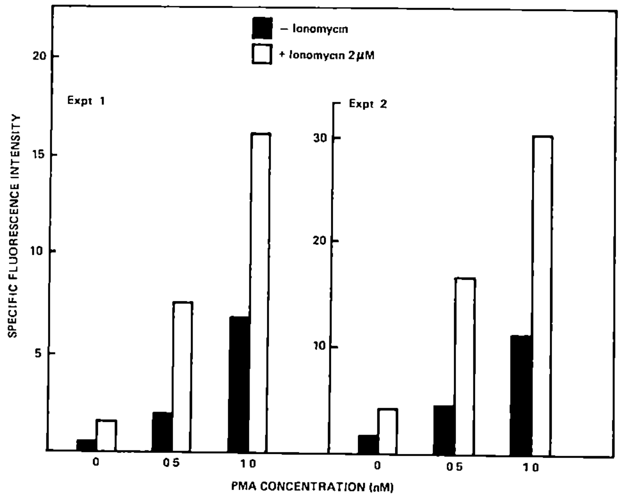

Fig 1. Synergism between ionomycin and submaximally stimulatory concentrations of PMA in up-modulating surface Mo3e expression on U-937 cells. U-937 cells (3 to 4 × 10^5/mL) were cultured for 24 hours in medium containing the indicated concentrations of PMA (X-axis) in the absence (■) or presence (□) of ionomycin 2 μmol/L. After culture, the cells were stained by indirect immunofluorescence for the expression of Mo3e as indicated on the Y-axis (specific fluorescence intensity as calculated in Materials and Methods). The fluorescence gain in experiments 1 and 2 was 1,050 and 1,200 mV, respectively. Fluorescence intensity of cells stained with a negative control antibody ranged from 0.27 to 0.33 (experiment 1) and from 0.31 to 0.43 (experiment 2).

DISCUSSION

In a variety of cellular systems, the effector response to external stimuli relies on signal transduction pathways that use "second messengers" to trigger effector kinases. Included among these second messengers are diacylglycerol and inositol trisphosphate (end products of receptor-mediated phosphatidylinositol hydrolysis), which activate protein kinase C and mobilize intracellular calcium ions, respectively.[4] In this report, we suggest that the surface expression of Mo3e may be among those cellular responses that are regulated by the generation of intracellular second messengers. We base this postulate on the following experimental observations:

(a) *PMA and other biologically active phorbol compounds stimulate the expression of Mo3e by monocytes, U-937 cells, and HL-60 cells.* Culture of monocytes and U-937 cells in medium containing PMA, PDB, or βPDD results in a marked increase in surface Mo3e expression that is easily detectable by immunofluorescence analysis within 24 hours (ref 1 and Tables 1, 2, and 5). HL-60 promyelocytic cells likewise become Mo3e positive after 72 hours of PMA exposure coincident with the expression of other monocytic features (Table 3). Surface Mo3e expression is not, however, an invariant marker of monocytic differentiation, since the majority of D_3-stimulated HL-60 cells remain Mo3e negative (Table 3) unless subsequently exposed to PMA (Table 4). Because PMA and related compounds can mimic the activity of endogenous second messenger 1,2-diacylglycerol by directly stimulating protein kinase C,[34-36] we speculate that surface Mo3e expression by human myeloid cells is a biological response to protein kinase C activation. Consistent with this notion is the fact that mezerein, although structurally dissimilar to PMA, activates protein kinase C[37] and, like PMA, induces surface Mo3e expression. Even more significant, surface Mo3e expression is also stimulated by exogenous membrane-permeable 1,2-diacylglycerol (L-α-1,2 dioctanoylglycerol [Table 5] or L-α-1-oleoyl-2-acetoyl-*sn*-3-glycerol) (cf references 27 and 28). Thus, these data suggest an association between the induction of Mo3e surface expression and protein kinase C activation by a variety of substitute second messengers that include PMA, PDB, βPDD, mezerein, and synthetic 1,2-diacylglycerol.

(b) *Surface Mo3e expression is stimulated or inhibited by exposure of U-937 cells to agents that modulate the concentration of intracellular or extracellular calcium ions.* Although the calcium ionophore ionomycin has little or no direct stimulatory effect on surface Mo3e expression, the combination of ionomycin 2 μmol/L with submaximally stimulatory concentrations of PMA (0.5 to 1.0 nmol/L) results in a synergistic increase in surface Mo3e density (Fig 1). Because ionomycin or calcium ionophore A23187 can mimic the effect of another second messenger, inositol tris-

Table 6. Inhibition of PMA-Stimulated Mo3e Expression by Calmodulin Inhibitors, Calcium Antagonists, and EGTA

Inhibitor	Inhibition of Mo3e Expression (%) (PMA Concentration) 0.5 nmol/L	1 nmol/L	5 nmol/L
Trifluoperazine (0.01 mmol/L)	77 ± 13	80 ± 11	53 ± 20
Chlorpromazine (0.02 mmol/L)	79 ± 11	79 ± 3	80 ± 1
Dibucaine (0.1 mmol/L)	97 ± 3	84 ± 14	73 ± 3
Nicardipine (0.02 mmol/L)	86 ± 12	77 ± 7	40 ± 13
TMB-8 (0.1 mmol/L)	64 ± 9	58 ± 15	52 ± 15
EGTA (1.5 mmol/L)	—	—	90 ± 2
EGTA (1.5 mmol/L) + $CaCl_2$ (2.0 mmol/L)	—	—	−1 ± 5
EGTA (1.5 mmol/L) + $MgCl_2$ (2.0 mmol/L)	—	—	87 ± 5

U-937 cells were cultured (3 × 10^5 cells per milliliter) for 22 hours in 1640-FCS containing the indicated concentrations of inhibitors and PMA (inhibitors added one hour before PMA). After culture, the cells were stained by indirect immunofluorescence for the expression of Mo3e. Specific immunofluorescence intensity for each population was determined (Materials and Methods), the percentage of inhibition of Mo3e specific immunofluorescence in cultures containing inhibitor relative to that of cultures with no inhibitor was calculated. Mean percentage ± SD of two to four separate determinations.

phosphate, in mobilizing cytosolic calcium ions from intracellular stores (endoplasmic reticulum),[4,29,30] we postulate that calcium may play a regulatory role in surface Mo3e expression. With this hypothesis in mind, we examined the effect of various agents that might antagonize the metabolism or influx of calcium ions. As shown in Table 6, we found that trifluoperazine, chlorpromazine, or dibucaine each consistently inhibited U-937 Mo3e surface expression stimulated by PMA. Although they are known as "calmodulin inhibitors" by virtue of their interference with the binding of calmodulin to kinase,[31,32] these compounds can also block the activity of calmodulin-independent protein kinase C by inhibition of the interaction between phospholipid and kinase.[5,6] Thus, the inhibitory effect of these agents on PMA-stimulated Mo3e expression could either suggest the involvement of calmodulin-dependent kinases or provide further evidence for the importance of protein kinase C activation in stimulating Mo3e expression.

Calcium influx from the extracellular compartment may also contribute to the regulation of surface Mo3e expression. The importance of extracellular calcium is suggested by the inhibitory effect of EGTA on Mo3e expression stimulated by PMA (Table 6), which in turn can be reversed by the addition of calcium, but not magnesium. Moreover, PMA-stimulated Mo3e expression is blocked by noncytotoxic concentrations of nicardipine and TMB-8, compounds that antagonize calcium mobilization and influx into mammalian cells.[7,8,33]

These observations are consistent with the role of the phosphatidylinositol signal transduction pathway in the regulation of surface Mo3e expression. According to current dogma, the binding of stimulatory ligands to plasma membrane receptors activates the guanine nucleotide regulatory protein system,[38–41] which stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, resulting in the generation of second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (see reference 4 for review). PMA and related compounds (as substitute second messengers that activate protein kinase C[33–35]) and ionomycin (that may duplicate the action of inositol trisphosphate) can bypass the initial membrane receptor-mediated events of signal transduction and directly trigger cellular effector responses. The physiologic significance of this pathway in the initiation of leukocyte effector responses to a variety of stimuli has been demonstrated in several recent reports. These include the metabolic responses of neutrophils (human, guinea pig, and rabbit) to *N*-formyl-L-methionyl-L-phenylalanine stimulation[39–42]; the proliferative response of T lymphocytes (murine and human) to soluble antigen, lectin, and interleukin 2[43–46]; the activation of B lymphocytes (murine and human) in response to anti-immunoglobulin stimulation[47,48]; the proliferation of murine bone marrow cells in response to interleukin 3[49]; and the activation of murine macrophages by gamma interferon or lipopolysaccharide.[50–52] Of particular note is the synergistic effect of PMA and calcium ionophore (ionomycin or A23187) acting as substitute second messengers in triggering T cell,[43,44,53] B cell,[54] and macrophage activation[50,55,56] (simulating the normal effector response to receptor-mediated stimuli) and, in certain systems, the inhibitory effect of calmodulin inhibitors,[57,58] EGTA,[42,43,55,56,58] and calcium influx antagonists.[42,55,58] Thus, the induction of surface Mo3e expression by agents that mimic the second messengers of the phosphatidylinositol transduction pathway is analogous to their effect on other leukocyte effector responses.

If PMA (and functionally related compounds) and ionomycin stimulate Mo3e expression by substituting for products of receptor-mediated phosphatidylinositol hydrolysis, what, then, is the physiologic stimulus for Mo3e expression? Because bacterial lipopolysaccharide is a potent inducer of surface Mo3e expression by peripheral blood monocytes[1] and has been shown to activate protein kinase C in murine macrophages and B lymphocytes,[52,59] lipopolysaccharide-stimulated Mo3e expression may be a cellular response that is triggered by products of the phosphatidylinositol transduction pathway. Bacterial endotoxin may therefore represent the physiologic "first signal" that induces the up-modulated expression of Mo3e. Consistent with this hypothesis is the fact that a selective inhibitor of protein kinase C activation (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H-7)[60] blocks the expression of Mo3e on monocytes cultured in medium containing *Escherichia coli* lipopolysaccharide (R.F.T. III, unpublished observations, March 1986).

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REFERENCES

1. Todd RF III, Alvarez PA, Brott DA, Liu DY: Bacterial lipopolysaccharide, phorbol myristate acetate, and murmayl dipeptide stimulate the expression of a human monocyte surface antigen, Mo3e. J Immunol 135:3869, 1985

2 Liu DY, Ree JY, Todd RF III: Characterization of Mo3e, a monocyte antigen involved in the response to migration inhibitory factor. Fed Proc 45:1115, 1986 (abstr)

3. Liu DY, Todd RF III: A monoclonal antibody specific for a monocyte-macrophage membrane component blocks the human monocyte response to migration inhibitory factor. J Immunol 137:448, 1986

4. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308:693, 1984

5. Mori T, Takai Y, Minakuchi R, Yu B, Nishizuka Y Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. J Biol Chem 255 8378, 1980

6. Wise BC, Glass DB, Chou CHJ, Raynor RL, Katoh N,

Schatzman RC, Turner RS, Kibler RF, Kuo JF· Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II. Substrate specificity and inhibition by various agents. J Biol Chem 257.8489, 1982

7 Terai M, Takanaka T, Maeño H· Inhibition of calcium influx in rabbit aorta by nicardipine hydrochloride (YC-93). Biochem Pharmacol 30:375, 1981

8. Chiou CY, Malagodi MH: Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N,N*-diethylamino) octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. Br J Pharmacol 53:279, 1975

9. Todd RF III, Nadler LM, Schlossman SF. Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126.1435, 1981

10. Todd RF III, Schlossman SF: Analysis of antigenic determinants on human monocytes and macrophages Blood 59:775, 1982

11. Ritz J, Schlossman SF: Utilization of monoclonal antibodies in the treatment of leukemia and lymphoma. Blood 59:1, 1982

12. Biondi A, Roach JA, Griffin JD, Todd RF III: Mb1, a plasma membrane antigen selectively expressed by U-937 cells. Blood 66.135, 1985

13. LeClair K, van Agthoven A, Terhorst C. A rapid method for isolation of antigenically active human cell surface antigens associated with β_2-microglobulin using a monoclonal antibody. J Immunol Methods 41 137, 1981

14. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937) Int J Cancer 17:565, 1976

15. Collins SJ, Gallo RC, Gallagher RE: Continuous growth and differentiation of human leukemic cells in suspension culture. Nature 270.347, 1977

16. Muirhead KA, Schmitt TC, Muirhead AR Determination of linear fluorescence intensity from flow cytometric data accumulated with logarithmic amplifiers. Cytometry 3·251, 1983

17 Todd RF III, Griffin JD, Ritz J, Nadler LM, Abrams T, Schlossman SF· Expression of normal monocyte-macrophage differentiation antigens on HL-60 promyelocytes undergoing differentiation induced by leukocyte-conditioned medium or phorbol diester. Leuk Res 5:491, 1981

18. Perussia B, Lebman D, Ip SH, Rovera G, Trinchieri G: Terminal differentiation surface antigens of myelomonocytic cells are expressed in human promyelocytic leukemia cells (HL-60) treated with chemical inducers. Blood 58.836, 1981

19. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant myeloid cells J Clin Invest 68:932, 1981

20. Graziano RF, Ball ED, Fanger MW. The expression and modulation of human myeloid-specific antigens during differentiation of the HL-60 cell line. Blood 58.836, 1983

21. McCarthy DM, San Miguel JF, Freake HC, Green PM, Zola H, Catovsky D, Goldman JM: 1,25-dihydroxyvitamin D_3 inhibits proliferation of human promyelocytic leukemia cells and induces monocyte-macrophage differentiation in HL-60 and normal human bone marrow cells. Leuk Res 7:51, 1983

22. Matsui T, Nakao Y, Kobayashi N, Kishihara M, Ishizuka S, Watanabe S, Fujita T. Phenotypic differentiation-linked growth inhibition in human leukemia cell by active vitamin D_3 analogues. Int J Cancer 23:193, 1984

23. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC· Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds, Proc Natl Acad Sci USA 75·2458, 1978

24 Breitman TR, Selnick SE, Collins SJ: Induction of differentiation of the promyelocytic leukemic cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 77.2936, 1980

25. Matsui T, Nakao Y, Koizuma T, Katakama Y, Fujita T: Inhibition of phorbol ester-induced phenotypic differentiation of HL-60 cells by 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine, a protein kinase inhibitor. Cancer Res 46:583, 1986

26. Olsson I, Gullberg U, Ivhed I, Nilsson K: Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1α, 25-dihydroxycholecalciferol. Cancer Res 43:5862, 1983

27. Ebeling JG, Vandenbark GR, Kuhn LJ, Ganong BR, Bell RM, Niedel JE. Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation Proc Natl Acad Sci USA 82 815, 1985

28 Davis RJ, Ganong BR, Bell RM, Czech MP· Sn-1,2-dioctanoylglycerol. A cell-permeable diacylglycerol that mimics phorbol diester action on the epidermal growth factor receptor and mitogenesis. J Biol Chem 260:1562, 1984

29 Streb H, Irvine RF, Berridge MJ, Schulz I. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. Nature 306:67, 1983

30 Joseph S, Thomas AP, Williams RJ, Irvine RF, Williamson JR. Myoinositol 1,4,5-trisphosphate. A second messenger for the hormonal mobilization of intracellular Ca^{++} in liver. J Biol Chem 259:3077, 1984

31. Weiss B, Levin RM. Mechanism for selectively inhibiting the activation of cyclic nucleotide phosphodiesterase and adenylate cyclase by antipsychotic agents Adv Cyclic Nucleotide Res 9·285, 1978

32. Wallace RW, Tallent EA, Cheung WY: Multifunctional role of calmodulin in biological processes Cold Spring Harbor Symp Quant Biol 46:893, 1981

33. Korchak HM, Rutherford LE, Weissman G: Stimulus response coupling in the human neutrophil. I Kinetic analysis of changes in calcium permeability. J Biol Chem 259:4070, 1984

34. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y: Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 257:7847, 1982

35. Niedel JE, Kuhn LJ, Vandenbark GR: Phorbol diester receptor co-purifies with protein kinase C. Proc Natl Acad Sci USA 80 36, 1983

36. Ashendel CL, Staller JM, Boutwell RK: Protein kinase activity associated with a phorbol ester receptor purified from mouse brain. Cancer Res 43:4333, 1983

37. Miyake R, Tanaka T, Tsuda T, Kaibuchi K, Kikkawa U, Nishizuka Y· Activation of protein kinase C by non-phorbol tumor promoter, mezerine. Biochem Biophys Res Commun 121:649, 1984

38. Brandt SJ, Dougherty RW, Lapetina EG, Neidel JE. Pertussis toxin inhibits chemotactic peptide-stimulated generation of inositol phosphates and lysosomal enzyme secretion in human leukemic (HL-60) cells Proc Natl Acad Sci USA 82:3277, 1985

39. Smith CD, Lane BC, Kusaka I, Verghese MW, Snyderman R: Chemoattractant receptor-induced hydrolysis of phosphatidyl inositol 4,5-bisphosphate in human polymorphonuclear leukocyte membranes. J Biol Chem 260·5875, 1985

40 Shefcyk J, Yassin R, Volpi M, Molski TFP, Naccache PH, Munoz JJ, Becker EL, Feinstein MB, Sha'afi RI: Pertussis but not cholera toxin inhibits the stimulated increase in actin association with the cytoskeleton in rabbit neutrophils: Role of the "G proteins" in stimulus-response coupling Biochem Biophys Res Commun 126:1174, 1985

41. Okajima F, Katada T, Ui M: Coupling of the guanine nucleotide regulatory protein to chemotactic peptide receptors in

neutrophil membranes and its uncoupling by islet-activating protein, pertussis toxin. J Biol Chem 260:6761, 1985

42. Verghese MW, Fox K, McPhail LC, Snyderman R: Chemoattractant-elicited alterations of cAMP levels in human polymorphonuclear leukocytes require a Ca^{2+}-dependent mechanism which is independent of transmembrane activation of adenylate cyclase. J Biol Chem 260.6769, 1985

43. Truneh A, Albert F, Golstein P, Schmitt-Verhulst AM: Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 313:318, 1985

44. Isakov N, Altman A: Tumor promoters in conjunction with calcium ionophores mimic antigenic stimulation by reactivation of alloantigen-primed murine T lymphocytes. J Immunol 135:3674, 1985

45. Ku Y, Kishimoto Y, Takai Y, Ogawa Y, Kimura S, Nishizuka Y: A new possible regulatory system for protein phosphorylation in human peripheral lymphocytes. II. Possible relationship to phosphatidylinositol turnover induced by mitogens. J Immunol 127:1375, 1981

46 Farrar WL, Anderson WB: Interleukin-2 stimulates association of protein kinase C with plasma membrane. Nature 315:233, 1985

47. Monroe JG, Niedel JE, Cambier JC: B cell activation. IV. Induction of cell membrane depolarization and hyper-I-A expression by phorbol diester suggests a role for protein kinase C in murine B lymphocyte activation. J Immunol 132:1472, 1984

48. Coggeshall KM, Cambier JC: B cell activation. VI. Effects of exogenous diglyceride and modulators of phospholipid metabolism suggest a central role for diacylglycerol generation in transmembrane signaling by mIg. J Immunol 134:101, 1985

49. Farrar WL, Thomas TP, Anderson WB: Altered cytosol/membrane enzyme redistribution on interleukin-3 activation of protein kinase C. Nature 315:235, 1985

50. Weiel JE, Adams DO, Hamilton TA: Biochemical models of γ-interferon action· Altered expression of transferrin receptors on murine peritoneal macrophages after treatment in vitro with PMA or A23187 J Immunol 134.293, 1985

51. Hamilton TA, Becton DL, Somers SD, Gray PW, Adams DO: Interferon-γ modulates protein kinase C activity in murine peritoneal macrophages. J Biol Chem 260:1378, 1985

52. Wightman PD, Raetz CRH: The activation of protein kinase C by biologically active lipid moieties of lipopolysaccharide. J Biol Chem 259:10048, 1984

53. Kaibuchi K, Takai Y, Nishizuka Y. Protein kinase C and calcium ion in mitogenic response of macrophage-depleted human peripheral lymphocytes. J Biol Chem 260:1366, 1985

54. Clevers HC, Versteegen JMT, Logtenberg T, Gmelig-Meyling J, Ballieux RE: Synergistic action of A23187 and phorbol ester on human B cell activation. J Immunol 135:3827, 1985

55. Smith BM, Sturm RJ, Carchman RA· Calcium modulation of phorbol ester-induced alteration in murine macrophage morphology. Cancer Res 43·3385, 1983

56. Somers SD, Weiel JE, Hamilton TA, Adams DO: Phorbol esters and calcium ionophore can prime murine peritoneal macrophages for tumor cell destruction. J Immunol 136:4199, 1986

57. Berger M, O'Shea J, Cross A: Role of calcium and calmodulin in expression of complement receptors. Fed Proc 43:1506, 1984 (abstr)

58. Onazaki K, Takenwa T, Homma Y, Hashimoto T. The mechanism of macrophage activation induced by Ca^{2+} ionophore. Cell Immunol 75:242, 1983

59. Rosoff PM, Cantley LC. Lipopolysaccharide and phorbol esters induce differentiation but have opposite effects on phosphatidylinositol turnover and Ca^{2+} mobilization in 70Z/3 pre-B lymphocytes. J Biol Chem 260:9209, 1985

60. Kawamoto S, Hikada H: 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H7) is a selective inhibitor of protein kinase C in rabbit platelets. Biochem Biophys Res Commun 125:258, 1984

Vaso-occlusion by Sickle Cells: Evidence for Selective Trapping of Dense Red Cells

By Dhananjaya K. Kaul, Mary E. Fabry, and Ronald L. Nagel

We have characterized the type of red cells from sickle cell patients that were trapped in the course of sickle-cell vaso-occlusion. In addition, the perfusion conditions (arterial perfusion pressure [Pa] and oxygen tension [PO$_2$]) leading to experimentally induced vaso-occlusion in the artificially perfused, innervated mesocecum microvascular preparation were determined. Microvascular obstruction was induced by decrease in Pa; the lower the Pa, the greater the peripheral resistance as well as the extent of obstruction. The cells involved in the obstruction were recovered by vasodilation (secondary to denervation) and increase in Pa. Densitometric analysis of density gradient–separated infused and trapped cells was supplemented with morphological analysis to ascertain the involvement of density classes as well as morphological types seen in oxy and deoxy sickle blood. The trapping phenomenon was sensitive to PO$_2$. Percentage of densest gradient classes, ie, fraction 3 (F3; mainly dense unsicklable SS discocytes [USDs]) and fraction 4 (F4; irreversibly sickled cells [ISCs] and the densest discocytes), showed a significant increase in trapping when perfusion was switched from oxy to deoxy perfusate. Morphological analysis revealed that unsicklable SS discocytes are more effectively trapped when deoxygenated. The deoxygenation of infused cells did *not* further change the percentage of ISCs trapped, suggesting that ISCs are equally capable of sequestration in the oxy and the deoxy states. The venous effluent showed a selective and significant depletion of dense cells (F4) and ISC counts at all Pa. We conclude that the progressive obstruction of the microcirculation by sickle cells involves selective sequestration of the densest classes of cells and that this mechanism might explain their partial disappearance during painful sickle cell crisis.

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THE PATHOPHYSIOLOGIC EVENTS involved in the development of painful crisis in sickle cell anemia are still largely undefined. However, an interesting objective sign of this event was recently reported by Fabry et al[1] who observed a reduction of high-density red cells (a 36% to 94% decrease was found if initial values are normalized to 100%) in the peripheral circulation during the course of painful crisis. After crises, a gradual recovery of dense cells was observed. In addition, in an earlier publication,[2] we suggested that variations in the local microvascular pressure gradient, particularly the occurrence of low pressure, might lead to preferential trapping of less deformable sickle cells.

The present study was designed to analyze the classes of cells involved in sickle cell-induced vaso-occlusion in an innervated, artifically perfused microcirculatory preparation. Oxygenated or deoxygenated sickle blood was allowed to obstruct a microvascular bed by decreasing the perfusion pressure. The cells involved in the obstruction were recovered by increasing the perfusion pressure and by denervation-induced vasodilation and were characterized by morphological and isopycnic gradient analysis. We find that of the cells trapped during vaso-occlusion, a disproportional percentage are represented by ISCs (irreversibly sickled cells) and USDs (unsicklable SS discocytes), the densest cells in the blood of the sickle cell anemia patient. In addition, we find that the deoxygenation of the perfused cells did not change the percentage of ISCs trapped, suggesting that even under oxygenated conditions, these cells are maximally capable of participating in the vaso-occlusive event. We conclude that the densest SS cells are indeed trapped in the areas of vaso-occlusion and that this phenomenon is likely to be the basis of the disappearance of these cells during painful crises.

MATERIALS AND METHODS

Preparation of cells. Fresh heparinized samples were obtained by venipuncture from three normal adults and from six sickle cell patients who were out of crises and had not received blood transfusion in the preceding 4 months. The blood samples were centrifuged (2,000 rpm) and the plasma and buffy coat removed. The red cells were then washed three times in normal saline and resuspended in bicarbonate Ringer solution of the following composition in millimoles per liter: NaCl, 118 0; KCl, 5.0; CaCl$_2$, 2.5; MgCl$_2$, 0 64; NaHCO$_3$, 27.0, plus 2 0% bovine albumin The osmolarity was adjusted to 290 to 300 mOsm with the use of a Microosmette osmometer (Precision Systems, Inc, Sudbury, Mass). The hematocrit (Hct) was adjusted to 2% for these experiments. Red cell suspensions (100 mL) and perfusion solution (bicarbonate Ringer, 2 0% bovine albumin) were equilibrated with gas mixtures containing O$_2$ (95% or 2 5%), 5.6% CO$_2$, and balance N$_2$ to achieve a pH of 7.35 to 7.40 and the desired pO$_2$ level

Artificially perfused innervated mesocecum vasculature. Hemodynamic studies and induction of vaso-occlusion under defined arterial perfusion pressure (Pa) were carried out in 16 artificially perfused, nerve intact mesocecum (mesoappendix) vasculature of the rat. For the nerve intact preparation, the procedure involves cannulation of the ileocolic artery and the vein under sodium pentobarbital anesthesia (30 mg/kg) as described by Baez et al[3] for isolated denervated mesocecum preparation Accompanying the ileocolic artery is the paravascular sympathic nerve; a loop of thread is gently passed around the nerve at a site closer to the cannulated artery, and the two ends of the thread are fastened together by an adhesive tape for a later easy access to the nerve during surgical denervation. Earlier in the procedure, cannulation of the femoral

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artery (for blood pressure monitoring) and tracheal cannulation (to minimize ventilatory problems) are performed.

After hemostatic ties of all vascular collection are achieved, the tissue is vascularly isolated but remains connected to the animal by the sympathetic nerve supply. The animal is then transferred to a special microscope stage and the mesocecum is gently spread on an optically clear Lucite block. Artificial perfusion with red cell suspensions and maintenance of the vasculature are done as described by Kaul et al.[2] The entire preparation is enclosed in a plastic capsule when required, except for the outlet of cannulas and the microscope objectives. The vasculature is initially perfused with Ringer's solution for 10 to 15 minutes to allow stabilization of the tissue and to clear the vasculature of the remaining blood cell elements of the host animal. For observation and measurements by intravital microscopic methods, we used a triocular Bausch and Lomb microscope equipped with a television camera and video monitor (Sony) as well as Panasonic video recorder (Model NV9300A)

Induction and hemodynamic monitoring of microvascular obstruction by sickle cells. Pa, which was rendered pulsatile (310 c/min) with a peristaltic pump, and venous outflow pressure (Pv) were monitored by means of Statham-Gould P-50 transducers (Gould, Oxnard, CA). Pv was kept at 3.8 mmHg and venous outflow (Fv) rate was monitored using a photoelectric drop-counter and expressed in milliliters per minute. The protocol used to induce microvascular obstruction with oxy and deoxy sickle red cell suspensions was essentially the same as described elsewhere.[2] In essence, the perfusion with red cell suspensions was performed using stepwise decrement in Pa (100, 80, 60, and 40 mmHg) Hemodynamic changes with the infusion of normal and HbSS red cells were determined in terms of peripheral resistance units (PRUs) as previously described[4] and expressed as $PRU = \Delta P/Q =$ mmHg/mL/min/g, where ΔP (mmHg) is the arteriovenous pressure difference and Q (mL/min/g) is the rate of Fv per gram of the tissue. Hemodynamic parameters, ie, Pa and Fv, were recorded on a physiograph (Model DMP-4B, Narco Biosystem Inc, Houston, Tex). The required level of tissue deoxygenation was achieved by equilibrating the suffusion solution (bicarbonate Ringer's plus 2% bovine albumin) with an appropriate gas mixture for 30 minutes and similarly equilibrating the perfusate with a gas mixture of the same composition for 30 minutes before initiating the experiment. The occurrence and the extent of the obstruction were confirmed by microscopic observation of the vascular bed. The duration of perfusion with oxy and deoxy SS cells was 25 to 30 minutes. The mean pO_2 of the partially deoxygenated sickle cell suspensions was 17.7 ± 2.3 mmHg at the arterial end and 24 ± 1 3 mmHg in the venous effluent Because the pO_2 of the arterial influx and venous efflux are nearly the same, the tissue pO_2 is within the same range.

Recovery of trapped sickle cells The perfusion was switched from cells to oxy Ringer's solution, and the trapped cells were obtained from the areas of microvascular obstruction by vasodilation secondary to the surgical denervation[5] and by increase in Pa to 150 to 200 mmHg (n = 9). Recovery of the trapped cells was accompanied by recovery in the Fv rate. By raising the Pa or denervation, 30 to 60 μL of packed cells were recovered that were sufficient for morphological and density gradient analysis.

In a series of experiments (n = 7), the venous effluent samples were collected at Pa of 80, 60, and 40 mmHg during infusion of sickle cells.

Density gradients and their evaluation Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia Fine Chemicals, Inc, Piscataway, NJ) and Stractan (arabino-galactan polysaccharide; St Regis Paper Co, West Nyack, NY) (prepared as described by Corash et al[6]) gradients were formed from a mixture of Percoll and Stractan as previously described.[1,7]

Differential morphological analysis of sickle cells. In a series of experiments with oxy and deoxy SS cell suspensions (n = 7), samples from infused cells and venous effluents were fixed in 10% neutral formaline for ISC counts. In selected experiments, aliquots of infused and trapped cells (oxygenated) were similarly fixed for counts of stress reticulocytes and ISCs Aliquots of infused and trapped cells were also deoxygenated (<10% HbO_2) and fixed in 10% neutral formaline for morphological differentiation of dense discocytes, reversible sickle cells, and ISCs. Scanning electron microscopy of cells was done as described before.[8]

Cells were classified as follows: in oxygenated preparations, stress reticulocytes were identified by their multilobulated appearance[8,9] and ISCs were identified by their axial ratio. In deoxygenated preparations, an additional class of cells could be identified. the USD, which had a bumpy surface, irregular discoid contour, and no projections with an axial ratio greater than 2:1.

A paired *t* test was used for statistical analysis of infused and trapped (or venous effluent) populations.

RESULTS

The microvascular obstruction in the innervated preparation by oxy and deoxy sickle cell suspensions was induced as described in Materials and Methods and was monitored as changes in the PRUs; the lower the Pa, the greater the PRUs as well as the extent of obstruction, in accordance with our earlier observations on denervated preparations.[2] The PRU values for AA cells showed slight elevation, which is not significant when compared with that for Ringer's solution (PRUs for Ringer's 5.4 ± 0.4 (mean ± SEM) and 6.2 ± 1.1, respectively, at Pa of 100 and 40 mmHg; PRUs for AA cells 6.4 ± 1.5 and 6.8 ± 1.2, respectively, at Pa of 100 and 40 mmHg). Perfusion with oxy SS red cells results in higher PRUs, particularly at low pressure (PRUs = 9.25 ± 2.0 and 17.60 ± 4.9, respectively, at Pa of 100 and 40 mmHg), which is indicative of the presence of some microvascular obstruction. When the vasculature is perfused with a partially deoxy sickle cell suspension, the increase in PRUs becomes increasingly pronounced (due to progressive obstruction) during stepwise reduction in Pa (PRUs = 12.4 ± 0.6 and 42.9 ± 11.7 at Pa of 100 and 40 mmHg, respectively, $P <$ .004 as compared with oxy SS PRUs), and the resulting obstruction is extensive. That highly deformable normal human (AA) red cells can traverse the rat mesoappendix without significantly elevating the peripheral resistance[2] supports the contention that mesoappendix is a suitable model for the human microvasculature. Furthermore, the relevant dimensions in the rat, such as the mean capillary diameter (4.5 μm), compare well with those of the human.

Trapped cells. The trapped cells from the obstructed areas were recovered by vasodilation and by increase in Pa (see Materials and Methods). The restoration of Fv rate strongly indicated that the majority of cells were eluted from the preparation. Furthermore, direct intravital observation of the mesocecum microvasculature revealed complete release of trapped cells in most instances. A Coulter counter was used to determine the number of cells in the perfusate and the effluent. From these values it was estimated that between 40 and 80 μL of packed SS cells were trapped in the average mesoappendix preparation and that at least 30 to 60 μL were recovered. These three observations indicate that the eluted cells are representative of the trapped cells.

A total of nine mesocecum preparations were perfused with cells from four sickle cell patients, and the infused cells were compared with the trapped cells either by density gradient analysis or by differential morphological counting or by a combination of both. The percentages of F3 (mainly dense USDs) and F4 (ISCs and densest discocytes) in both the infused red cell suspension and the red cells trapped in the microcirculation in the process of vaso-occlusion for experiments in which cells are infused either fully liganded (oxy) or deoxygenated (PO_2, 20 mmHg) were determined by Percoll-Stractan isopycnic gradients (Table 1, Fig 1). The results demonstrate that in all experiments, the percentage of F3 and F4 is invariably higher in the trapped cell category. Statistical analysis reveals that the differences in percentages of trapped F3 and F4 are significant at $P < .03$ and $P < .0016$, respectively, if the red cells were infused in the oxygenated state (Table 1). When cells were infused in the deoxygenated state, both F3 and F4 (Table 1) showed significant increases in trapped cells at the $P < .0012$ and $P < .0004$ levels, respectively. Also, as depicted in Fig 1, the ratios of trapped to infused F3 and F4 populations undergo significant increases (F3: oxy 1.3 ± 0.27, mean ± SD, deoxy 2.0 ± 0.42, $P < .036$; F4: oxy 1.3 ± 0.08, deoxy 2.2 ± 0.67, $P < .033$) when the infused sickle cell suspensions are deoxygenated (Fig 1). We conclude that the densest cells in the whole blood of sickle cell anemia patients are disproportionately found among the cells trapped during vaso-occlusion.

In three of these experiments the cells infused and cells trapped in the microcirculatory bed were characterized morphologically. If the cells were counted while oxygenated, only ISCs, stress reticulocytes, and discocytes were identifiable. A significant increase in the ISC count is observed in the trapped cells, both (and to similar extent) when the cells infused were oxygenated and deoxygenated (Table 2, Fig 2). In the oxy infusion, only stress reticulocytes, which have bumpy and lobulated shapes,[8,9] are overrepresented among the trapped cells. If aliquots of the infused cells and the trapped cells collected after the perfusion experiments were deoxygenated (for the sole purpose of counting), the cells that can now be identified are USDs (granular-surfaced discocytes after deoxygenation) and sickled cells. The cells that retain their smooth discoid morphology in extreme deoxy conditions are likely to contain a large percentage of HbF (fetal hemoglobin) (Fig 2). The data in Table 2 demonstrate that the USDs are also increased among the trapped cells but to a greater extent when the cells are infused in the deoxy state. Further, the ratio of trapped to infused USDs shows a significant difference with deoxygenation of infused sickle cell suspensions (oxy 1.2 ± 1.16, mean ± SD; deoxy 1.8 ± 1.1, $P < .014$), while the differences for ISCs are statistically not significant (oxy 1.6 ± 1.2; deoxy 1.48 ± 1.3, $P > .75$) (Fig 3).

Table 1. F3 and F4 in Cells Infused and Cells Trapped

	F3 (%)		F4 (%)	
Perfusion	Infused	Trapped	Infused	Trapped
Oxy perfusion				
Experiment 1	4.2	4.8	5.2	7.5
2	9.1	15.6	10.7	14.1
3	15.8	19.5	25.6	31.0
4	17.6	18.8	17.2	22.2
5	7.2	10.9	9.8	13.2
Mean	10.8	13.9	13.7	17.6
SD ±	5.7	6.1	7.9	9.1
P		<0.03		<0.0016
Deoxy perfusion				
Experiment 1	5.1	12.2	4.8	12.6
2	16.2	22.9	21.6	31.4
3	4.4	9.2	5.3	15.4
4	7.2	14.9	9.8	18.1
Mean	8.2	14.8	10.4	19.4
SD <	5.5	5.9	7.8	8.3
P		<0.0012		<0.0004

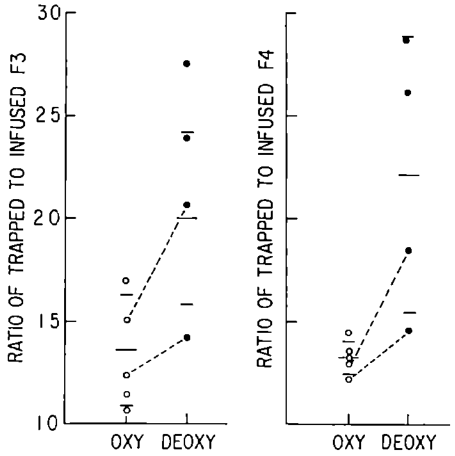

Fig 1. Ratios of trapped to infused F3 (mainly dense unsicklable SS discocytes) and F4 (mainly ISCs and very dense discocytes) cells in mesocecum preparation perfused with oxy and deoxy sickle cell suspensions. Note the increase in the ratio when the infused cells are deoxygenated. The paired data points (broken lines) represent the results of oxy and deoxy perfusion experiments done in the same preparation. Horizontal bars indicate mean ± SD.

Venous effluents. Finally, another set of experiments performed on seven preparations made use of Percoll-Stractan isopycnic gradient analysis and morphological counting of ISCs in the venous effluent samples from perfused mesoappendix preparations that had suffered vaso-occlusion by the infusion of oxy or deoxy SS cell suspensions. As expected, the differences in percentage of F4 between the infused cells and the effluent were small (infused cells, mean 19.5 ± 8.5 SD; effluent, 16.2 ± 6.6; $P < .015$) (due to the small number of cells involved in vaso-occlusion), but in each of seven experiments, the percentage of F4 in the effluent was invariably lower than in the infused cells. A significant decrease in the percentage of ISCs (as determined morphologically) occurred as Pa was reduced ($P < .007$ and $P < .0005$ at Pa of 80 and 40 mmHg, respectively) (Fig 4). This decrease was irrespective of the ligand state of the infusion.

DISCUSSION

The experiments described here demonstrate that in the course of vasoocclusion, the densest cells in the blood of sickle

Table 2. Differential Cell Counts in Control (Infused) and Trapped Samples

Perfusion	Stress Reticulocytes (%) Infused	Stress Reticulocytes (%) Trapped	USDs (%) Infused	USDs (%) Trapped	ISCs (%) Infused	ISCs (%) Trapped
Oxy perfusion						
1 (RS)	2.3	4.7	18.5	25.0	16.4	22.0
2 (EM)	0.5	2.0	13.0	13.5	12.5	21.5
3 (TP)	1.5	6.0	8.4	9.8	8.0	13.0
Mean	1.4	4.2	13.3	16.1	12.3	18.8
SD ±	0.9	2.0	5.1	7.9	4.2	5.1
P		<0.05		<0.15		<0.02
Deoxy perfusion						
1 (RS)	2.2	3.0	16.0	29.0	18.2	22.4
2 (EM)	1.0	0.7	13.0	22.0	12.0	17.0
3 (TP)	2.0	2.5	7.3	13.7	7.0	12.5
Mean	1.7	2.1	12.1	21.6	12.4	17.3
SD ±	0.6	1.2	4.4	7.7	5.6	4.96
P		<0.25		<0.02		<0.003

cell anemia patients (F3 and F4 of the Percoll-Stractan isopycnic gradient separation) are selectively trapped. In morphological terms, these fractions are composed of ISCs and a category of cells recently described,[8] the dense USDs. However, these two categories of dense cells do not behave identically in the microvasculature. The ISCs are trapped to an equal extent whether the perfused blood is oxygenated or deoxygenated, while the USDs are more susceptible to trapping when the infused blood is deoxygenated. This suggests that the ISC is as susceptible to sequestration during vaso-occlusion, when its cytosol is largely devoid of polymer, as it is when polymer is present. On the other hand, the trapping of USDs is further increased after the perfusate is deoxygenated.

The SS stress reticulocyte,[8,9] a cell that is shaped differently than a biconcave disk when oxygenated (it is highly irregular and lobulated), is also overrepresented among trapped cells when the infused blood is oxygenated. This is not surprising, as this cell is less deformable due to its peculiar shape.[10] After deoxygenation, it becomes hemodynamically indistinguishable from reversibly sickled cells and loses its special status.[8]

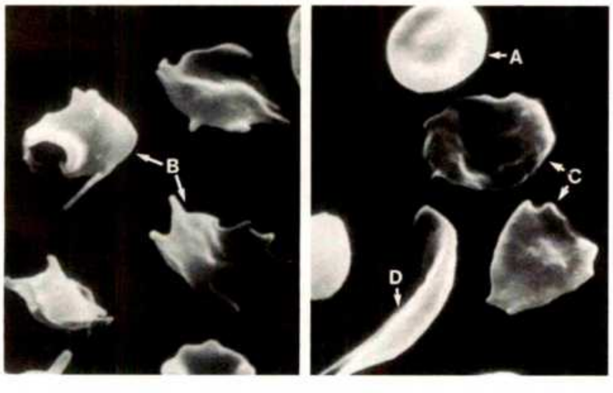

Fig 2. Scanning electron micrograph of cells deoxygenated in order to differentiate the major morphological types of sickle cells. [A, high fetal hemoglobin (HbF) cell (smooth discoid morphology); B, sickled cells (reversible discocytes); C, dense unsicklable SS discocytes (USDs) with irregular or granular contour and projections with an axial ratio of less than 2:1; D, ISCs].

We conclude that the less deformable cells are sequestered at the site of sickle cell-induced vaso-occlusion. It is important to understand that these experiments were not designed to determine which cells are responsible for initiating vaso-occlusion. They only show which cells are found among the cells trapped in the areas of vaso-occlusion after the episode has been allowed to complete its course. Other experiments have to be designed to dissect the sequences of events involved: that is, are the densest cells per se involved in initial obstruction, or are they trapped subsequent to the precapillary obstruction after red cell aggregation has extended in a retrograde manner toward the arteriolar side? Under these conditions, the flow in the partially blocked arterioles might bias the trapping in favor of the least deformable cells. We favor the second possibility because initial obstruction most likely involves a small number of cells, as has been observed by direct videoscanning of perfused microvascular beds.[11-14] Nevertheless, the findings reported here strongly buttress the

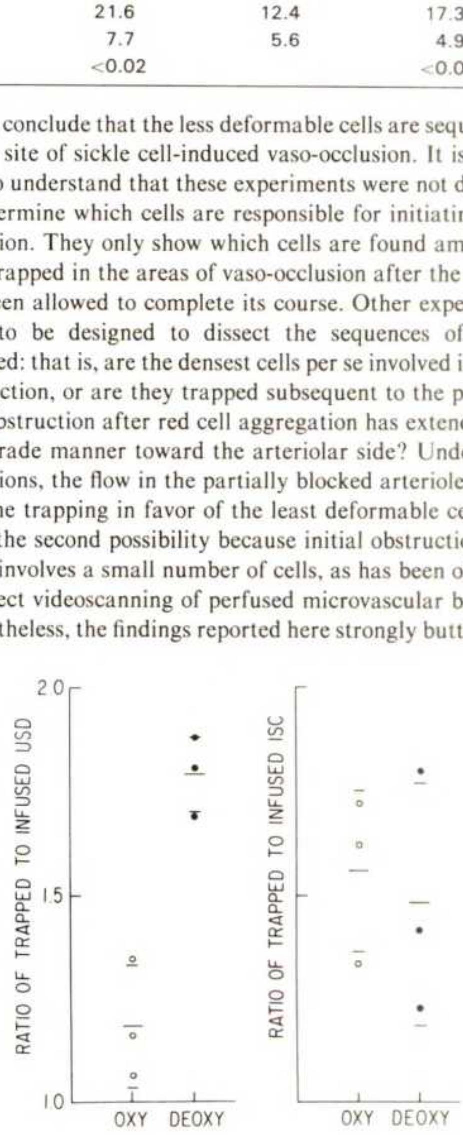

Fig 3. Ratios of trapped to infused unsicklable SS discocytes (USDs) and ISC counts in mesocecum preparation perfused with oxy and deoxy sickle cell suspensions. There is a dramatic increase in the ratio of USDs trapped when the vasculature is perfused with deoxy sickle cell suspensions. In contrast, the ratio of ISCs trapped is unchanged on deoxygenation. Horizontal bars indicate mean ± SD.

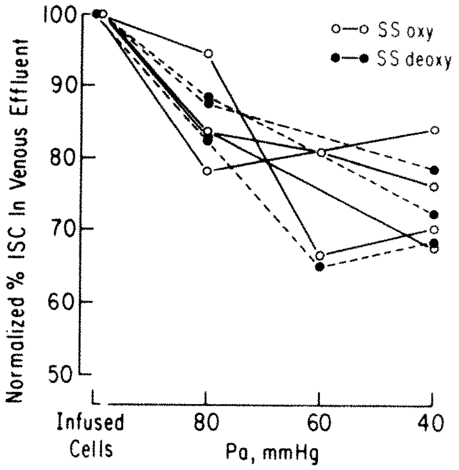

Fig 4. Plot of relative decrease (%) in the counts of ISCs in the venous effluents obtained at different Pa. The control values (infused cells) were normalized to 100%. The trapping of ISCs is enhanced as Pa is lowered to 40 mmHg.

concept that selective sequestration of dense cells during vaso-occlusion is a likely explanation for the disappearance of these cells during the painful crises suffered by sickle cell anemia patients.

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REFERENCES

1. Fabry ME, Benjamin L, Lawrence C, Nagel RL: An objective sign in painful crisis in sickle cell anemia: The concomitant reduction of high density red cells. Blood 64:559, 1984

2. Kaul DK, Nagel RL, Baez S: Pressure effects on the flow behavior of sickle (HbSS) red cells in isolated (ex-vivo) microvascular system. Microvasc Res 26:170, 1983

3. Baez S, Lamport H, Baez A: Pressure effects of living microscopic vessels, in Copley A, Stainby G (eds): Flow Properties of Blood and Other Biological Systems. London, Pergamon, 1960, p 122

4. Green HD, Rapela CE, Conard MD: Resistance (conductance) and capacitance phenomena in terminal vascular beds. Handb Physiol 2 (Sect 2, Circulation): 935, 1963

5. Baez S: Response characteristics of perfused microvessels to pressure and vasoactive stimuli. Angiology 12:452, 1961

6. Corash LM, Piomelli S, Chen HC, Seaman C, Gross E: Separation of erythrocytes according to age on a simplified density gradient. J Lab Clin Med 84:147, 1974

7. Fabry ME, Nagel RL: The effects of deoxygenation on red cell density: Significance for the pathophysiology of sickle cell anemia. Blood 60:1370, 1982

8. Kaul DK, Fabry ME, Windisch P, Baez S, Nagel RL: Erythrocytes in sickle cell anemia are heterogeneous in their rheological and hemodynamic characteristics. J Clin Invest 72:22, 1983

9. Coulombel L, Tchernia G, Mohandas N: Human reticulocyte maturation and its relevance to erythropoietic stress. J Lab Clin Med 94:467, 1979

10. Leblond PF, Coulombe L: The measurement of erythrocyte deformability using micropore membrane. J Lab Clin Med 94:133, 1979

11. Klug PP, Lessin LS: Microvascular flow of sickle erythrocytes. A dynamic morphological study. Blood Cells 3:263, 1977

12. LaCelle PL: Oxygen delivery to muscle cells during capillary vascular occlusion by sickle erythrocytes. Bloods Cells 3:273, 1977

13. Baez S, Kaul DK, Nagel RL: Microvascular determinants of blood flow behavior and HbSS erythrocyte plugging in microcirculation. Blood Cells 8:127, 1982

14. Lipowsky HH, Usami S, Chien S: Human SS red cell rheological behavior in the microcirculation of cremaster muscle. Blood Cells 8:113, 1982

Phenotype of Early Erythroblastic Leukemias

By J.L. Villeval, P. Cramer, F. Lemoine, A. Henri, A. Bettaieb, F. Bernaudin, Y. Beuzard, R. Berger, G. Flandrin,
J. Breton-Gorius, and W. Vainchenker

Nine cases of early erythroblastic leukemia, unidentified by usual criteria, have been diagnosed using a panel of antibodies. Three cases arose in patients with Down's syndrome, one in a patient with therapy-related leukemia, and four patients were in blast crisis of chronic myeloid leukemia; only one case arose de novo. Blast cells could be assigned to two main stages of erythroid differentiation: (1) presence of all erythroid-specific proteins in two patients, a phenotype corresponding to an immature erythroblast; (2) absence of the erythroid markers such as glycophorin A and spectrin in the presence of carbonic anhydrase isoenzyme I, ABH group antigens, and the antigen defined by FA6 152 monoclonal antibody in six patients, a phenotype related to a late erythroid progenitor (CFU-E). One patient had an intermediate phenotype. All patients except one demonstrated a megakaryocytic component. In three patients, chromosomal abnormalities were present, detected both in blasts and in erythroid colonies. In conclusion, these findings indicate that (a) most "cryptic erythroleukemias" are blocked at a "CFU-E-like" stage of differentiation, (b) it may be a frequent event in Down's syndrome and chronic myeloid leukemia, and (c) these erythroleukemias are phenotypically heterogenous.

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ON THE BASIS of conventional criteria, erythroleukemia represents an uncommon disease, ie, less than 5% of the leukemias. At least some of these leukemias may not be true erythroid neoplasms, since frequently the marrow becomes progressively invaded by myeloblasts.[1] In some cases of M$_6$ leukemia[2] the erythroblasts originate from residual normal marrow cells.[3] This low incidence of erythroleukemia in humans seems strange, since myeloproliferative diseases, especially chronic myeloid leukemia (CML), originate in a pluripotent stem cell.[4] The concept of "cryptic erythroleukemia" or of an "early" erythroblastic leukemia (leukemic cells blocked at an early stage of the erythroid differentiation and not morphologically identifiable by the usual criteria) has emerged. Most authors have used antibodies against glycophorin A (GPA) as a probe for the diagnosis of these "early" cases. Andersson et al[5,6] used a polyclonal antibody against GPA and suggested that "cryptic erythroleukemia" is frequent among M$_1$-acute myeloid leukemia (AML), relapse of acute lymphoblastic leukemia (ALL), and blast crisis of CML.[7] Other authors used several monoclonal antibodies (MoAbs) against GPA and concluded that erythroleukemia is uncommon.[8-11] This low incidence of erythroleukemia could be due to the fact that GPA detected by MoAbs was not a sufficiently early marker of erythroid differentiation. Indeed, GPA detected by these MoAbs is not expressed on the membrane of erythroid progenitors, ie, CFU-E and BFU-E.[12]

In the present study, we have investigated acute leukemia with a panel of MoAbs and polyclonal antibodies, including an anti-carbonic anhydrase I (CAI) and FA6-152 MoAb.[13] These two antibodies recognize antigens that are expressed before GPA during normal erythropoiesis.[13,14] Nine cases of erythroblastic leukemias could be diagnosed; the anti-GPA MoAbs only identified two of these cases.

MATERIALS AND METHODS

Leukemia samples. Over a period of 3 years, 310 cases of acute leukemia were investigated in our laboratory by immunological markers and by electron microscopy. Mononuclear cells were separated by Ficoll-metrizoate gradient density centrifugation (Lymphoprep, Nyegaard, Oslo, d:1077)[15] and subsequently investigated.

The nine cases presently reported were diagnosed among 120 cases of poorly differentiated acute leukemia. Patients 5 through 8 were identified as part of systematic investigations of the phenotype of CML blast crisis. The others (patients 1 through 4 and 9) were referred to our laboratory because they could not be classified according to the French-American-British criteria.[2] In two of these cases (patients 1 and 3), the erythroid origin of the blast cells was strongly suspected based on the morphological features, ie, the intensely basophilic cytoplasm.

Panel of antibodies and lectin. Details of antibodies used in this study are given in Table 1. During the first two years, the panel of MoAbs did not include early erythroid markers other than the LICR-LON-R10 MoAb[16]; During the last year, two new immunological markers that recognize early erythroid markers[13,14] were added to the previous panel. For blood group A patients, the A antigen was investigated using the Helix pomatia (HPA) lectin directly coupled to fluorescein (Industrie Biologique Française, Villeneuve la Garenne, France). Among 30 cases of non-erythroid leukemia, we have detected this antigen in only one case of megakaryoblastic leukemia.

The anti-CAI antibody and the FA6 152 MoAb were recently characterized using normal and leukemic cells.[13,14] Eighty representative cases of AML, ALL, or CML were screened with the anti-CAI antibody. CAI was not observed in non-erythroid hematopoietic cells with the exception of three cases in which this isoenzyme was detected in platelets. Further investigation of the occurrence of CAI in the megakaryocytic lineage has shown the absence of this enzyme in the platelets of 30 normal donors and 50 cases of myeloproliferative disorders. The FA6 antibody was used to screen more than 110 cases of AML, ALL, and blast crisis of CML. This antibody did not bind to ALL cells or M$_1$, M$_2$, and M$_3$ acute

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Table 1. Antibodies Used

Designation	Monoclonal	Specificity	References
Granulomoncytic markers			
80 H 5	+	SSEA-1 antigen (Promyelocyte → polymorph) (Monoblast → promonocyte)	17
MO_2	+	Monocytes	18
Anti-My 7	+	Immature myeloid cells, monocytic series	19
Anti-My 9	+	Immature myeloid cells, monocytic series	20
Anti-myeloperoxidase	−	Myeloperoxidase (Myeloblast → polymorph) (Monoblast → monocyte)	21
Platelet markers			
AN 51	+	Platelet glycoprotein Ib (Promegakaryoblast → platelet)	22
J 15	+	Glycoprotein IIb-IIIa? (Promegakaryoblast → platelet)	23
5 G 11	+	Thrombospondin (Promegakaryoblast → platelet)	24
Anti vWF	−	vWF (Promegakaryoblast → platelet)	Dako (Copenhagen) 25
B Cell markers			
B 1	+	Pre B → lymphocyte	Coulter clone (Hialeah, Fla) 26
J 5	+	Common ALL antigen	Ortho (Westwood, Mass) 27
T cell marker			
T 11	+	E rosette receptor	Ortho (Westwood, Mass) 28
Progenitor markers			
20-6	+	HLA-DR	29
Anti-My 10	+	All progenitors except CFU-E Immature myeloid cells	30
Erythroid markers			
LICR-LON-R10 and R 18	+	Glycophorin A (Proerythroblast → erythrocyte)	16
Anti-glycophorin A	−	Glycophorin A	5
LICR-LON-R6A	+	Band 3 ? (Erythroblast → erythrocyte)	16
	−	Band 3	
CA 5	+	β Chain of spectrin (Proerythroblast → erythrocyte)	
	−	α and β Chain of spectrin	
FA6 152	+	A part of BFU-E, CFU-E → erythroblast Megakaryocyte → platelet, monocyte	13
Anti-Gerbich	+	(CFU-E → erythrocyte), glycophorin C	Centre National de Transfusion Sanguine (Paris) 31
Anti-globin chain	−	γ-Globin chain	32
Anti-hemoglobin	−	Hemoglobin	Cappel (Cochranville, Pa)
Anti-CAI	−	CAI (CFU-E → erythrocyte)	14

leukemia cells. Positive cells were identified in leukemias with a monocytic component such as some M_5 and M_4 and part of the megakaryocytic leukemias. In all these cases, the monocytic markers recognized by 80H5,[17] My7,[19] My9,[20] anti-HLA-DR[29] MoAbs, or the megakaryocytic markers identified by AN51[22] or J15[23] MoAbs were detected on these non-erythroid FA6-positive cells.

Indirect immunofluorescence. Leukemic cells to be characterized were used fresh or thawed when further investigation was needed. An indirect immunofluorescence technique, including double-staining experiments, was used on fixed or unfixed cells.[14] For routine immunodiagnostic procedure, the panel of surface markers listed in Table 2 was used as well as three antibodies recognizing cytoplasmic proteins, ie CAI, myeloperoxidase (MPO), and von Willebrand factor (vWF). When the phenotype was compatible with that of an erythroid precursor (positive cells with FA6 MoAb, the anti-CAI antibody, and in some with anti-GPA MoAbs), the other erythroid markers listed in Table 3 were also evaluated.

Acetylcholinesterase. Acetylcholinesterases (AChE) were cytochemically revealed by the technique of Jackson.[33]

Clonogenic assays. Clonogenic assays for the different types of hematopoietic progenitors were performed by the methylcellulose techniques.[34] The stimulating factors were 1 IU/mL porcine Epo (26

Table 2. Cell Marker Results

						Cells Staining (%)												
Patient No.	Primary Syndrome	Age (yr)	Sex	Leukocytes (/µL)	Blasts	20-6	My 10	My 9	My 7	80 H 5	MO_2	B1	J5	T11	AN 51	J15	FA6 152	R 10 R 18
1	Down's syndrome	3	M	15,000	65	1	<1	<1	<1	<1	<1	<1	0	11	3	7	70	58
2	Down's syndrome	2	M	12,000	60	15	0	ND	0	1	1	0	0	23	3	3	66	1
3	Down's syndrome	1.5	M	10,000	50	3	0	<1	<1	<1	3	8	0	14	0	0	70	0
4	Therapy-related leukemia (Hodgkin's disease)	49	M	6,800	50	ND	ND	ND	ND	8.5	ND	ND	0	0	19	22	ND	26
5	CML	34	M	*	63	2	4	ND	<1	<1	<1	0	0	2	3	3	70	6
6	CML	58	F	103,000	19	0	0	0	0	54	0	0	0	0	2.5	3	46	4
7	CML	17	M	82,000	23	5	5	4	5	42	0	0	0	<1	16	17	52	<1
8	CML	50	F	71,000	56	0	0	0	0	0	0	<1	0	2	1	13	78	3
9	—	58	M	52,000	47	2	2	2	2	7	2	1		2	1	1	70	<1

The results are expressed in percentage of fluorescent-labeled cells isolated by Ficoll-metrizoate density centrifugation. The percentage of blasts was determined on the May-Grünwald-Giemsa on standard smears. In patients 6 and 7, the Ficoll-metrizoate density centrifugation has led to a twofold enrichment in blast cells as a consequence of the presence of numerous mature myeloid cells. ND, not determined; *, marrow specimen.

IU/mg of protein, Centre National de Transfusion Sanguine, Paris) and phytohemagglutinin-leukocyte–conditioned medium (PHA-LCM).[35]

Cytogenetics. Chromosome analysis was carried out on unstimulated blood cells and bone marrow cells after short-term culture (24 to 48 hours) in all cases and on colonies in three cases (5, 6, and 9). In addition, chromosome analysis was carried out after 2 months in liquid culture in patient 6. R bands using Giemsa after heating (RHG) and G bands using Wright staining were performed, and the international nomenclature[36] was used for the classification of the chromosomes. Metaphases from individual colonies were analyzed for three patients using a slight modification of the technique of Dubé et al.[37]

RESULTS

Immunofluorescence assays. Among 120 cases of leukemias, nine cases had a major erythroid component. In two of these cases (patients 1 and 4), the diagnosis could be made with anti-GPA MoAbs. In the seven others, the diagnosis depended on demonstration of cells that bound the FA6 MoAb and the anti-CAI antibody. Only 0% to 6% of cells from these patients were labeled by the anti-GPA MoAbs (Table 2).

In order to accurately determine the phenotype of the blasts, the nine leukemic samples were further investigated with the other erythroid markers (Table 3). Patient 4 exhibited two populations of blast cells distinguished by their size. The very large GPA- and CAI-positive blasts also contained HbF, whereas the small blast cells expressed platelet glycoproteins and vWF, suggesting an origin from the megakaryocytic lineage. In patient 1, the blast cells exhibited nearly all erythroid markers.

Among the seven other patients (patients 2, 3, and 5 through 9), one (patient 2) exhibited a peculiar phenotype. AChE, HbF, and glycoprotein band 3 were detected in the same cell population identified by the GPA MoAbs. In contrast, the anti-GPA polyclonal antibody bound to 40% of the blast cells. In addition, spectrin and Gerbich and A antigens were detected in the majority of these blasts. The other six patients (3 and 5 through 9) had a somewhat heterogenous phenotype. Only Gerbich or A antigen was clearly expressed in four cases. In the three blood group A patients (patients 3, 7, and 9), HPA lectin bound to about half of the leukemic cells, and in patient 8, Gerbich antigen was expressed in the same proportion of cells.

Double-labeling experiments were performed in four patients with CML blast crisis. In patients 6 and 7, a marked myeloid component was present but only included mature myeloid cells easily identifiable by light microscopy. In all cases, the myeloid and megakaryocytic cells, respectively labeled by 80H5, My9, 5G11, or J15 MoAbs, did not express

Table 3. Erythroid Cell Marker Results

		GPA		Spectrin								
Patient No.	FA6 152 (%)	MoAb (%)	Poly Ab (%)	MoAb (%)	Poly Ab (%)	R6A	BAND 3 Poly Ab (%)	CA I (%)	Hb F (%)	AChE (%)	Gerbich Antigen (%)	A Antigen
1	70	58	82	83	83	5	45	90	3	5	60	80
2	66	1*	35*	70*	75*	0*	1*	87*	0	0	60	90*
3	70	0	4	1	4	0	1	73	1	1	0	30
4	ND	26	ND	ND	ND	ND	ND	26	23	ND	ND	†
5	70	6	8	2	8	3	3	91	<1	5	4	†
6	46	4	4*	1*	4*	ND*	4*	62*	5*	4*	ND	†
7	52	<1	<1	ND	5	ND	1	25	1	1	<1	35
8	78	3	4	ND	5	0	2	62	<1	<1	30	†
9	70	1	4	<1	11	<1	ND	80	<1	<1	ND	34

The results were determined after fluorescent labeling except for the investigation of acetylcholinesterase (AChE), which was revealed by a histochemical technique. All samples were blood cells with the exception of patient 5, from whom marrow cells were obtained.

*These results have been determined from a second sample, obtained a few days later; for patient 6, in this second sample, 23% of the cells expressed MPO and 7%, vWF.

†These patients did not belong to blood group A.

CAI. All 80H5-positive cells expressed MPO. Labeling with the antithrombospondin (5G11) and the anti-vWF was only observed in cells stained by J15. This labeling resulted in a granular appearance, suggesting that most of these cells were hypoploid mature megakaryocytes (micromegakaryocytes).[25] In addition, platelets from patients (3, 6, 7, and 9) were examined for their content of CAI; this isoenzyme could not be detected.

Finally, it is noteworthy that in all cases presently described, a constant lack of labeling was observed with the My9, My10, and the anti-HLA-DR MoAbs. In patient 2, the anti-HLA-DR MoAb stained 15% of the cells, but binding did not correspond to erythroid cells because CAI-positive cells were not stained by the anti-HLA-DR antibody in double-labeling experiments.

Morphological characterization. The morphology of these blasts was reexamined after diagnosis with the immunological markers. In all patients, erythroid blast cells had a peculiar and identical morphology (Fig 1B). They were characterized by a medium to large size ($15\ \mu$), an extremely basophilic cytoplasm with the exception of a zone localized near the nucleus, and the presence of several peroxidase-negative azurophil granules or small vacuoles in the Golgi zone. The nucleus was large with one or two nucleoli.

Karyotypic analysis in short-term cultured cells and erythroid colonies. Using standard short-term cultures, clonal acquired chromosomal abnormalities were observed in all patients except patient 3, in whom only a constitutional trisomy 21 was found. Karyotypic studies of colonies derived from clonogenic assays were obtained in patients 5, 6, and 9. In patient 5, erythroid colonies were formed. All mitoses of leukemic cells were hyperdiploid (56 to 64 chromosomes) in 24-hour blood cell culture. All had a Philadelphia (Ph^1) chromosome and extra chromosomes with some cell-to-cell variation. A similar pattern of chromosomal changes was found in erythroid colonies from bone marrow, but only diploid mitoses with Ph^1 chromosome were found in other colonies, except one cell in a blood granulomonocytic colony (Table 4). In patient 9, no granulomonocytic colonies could be grown. Erythroid colonies comprising up to 400 cells were obtained in the presence or absence of added Epo. The cells composing these colonies expressed all the specific erythroid proteins, including hemoglobin. Multiple chromosomal abnormalities were found in unstimulated 48- and 78-hour blood cell cultures and in erythroid colonies except in four mitoses without markers (Table 4). A marker ? t (1;15) (p.21; q36) was present in all abnormal mitoses either from short-term blood cultures or from erythroid colonies. Other abnormalities, such as monosomies 17 and 10, were also common but not always observed, with some variation from one cell type to another. The majority of the cells were hypodiploid.

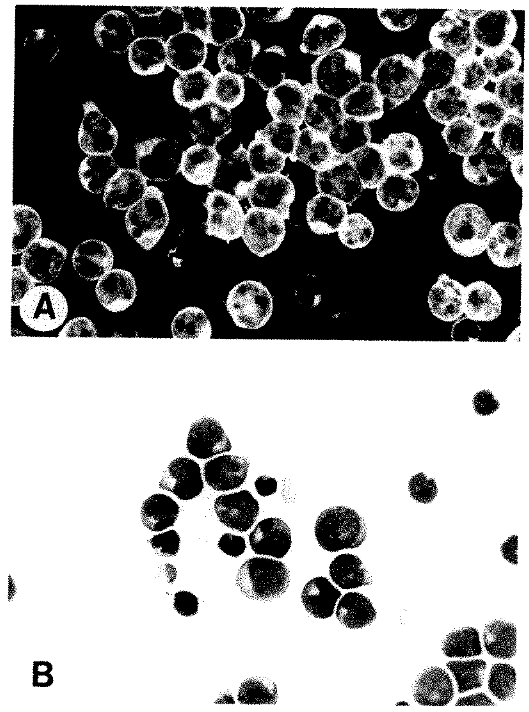

Fig 1. Blast cells from patient 2. (A) Labeling with the anti-CAI antibody. A marked staining is observed in all blast cells. (B) Morphology of the blast cells after May-Grünwald-Giemsa staining. The blasts are characterized by a medium size, an extremely basophilic cytoplasm, and an often irregular nucleus with one or two large nucleoli.

In patient 6, two types of erythroid colonies were obtained in the presence of Epo, either "morphologically normal" erythroid colonies similar to BFU-E colonies or atypical colonies comprising dispersed cells. The erythroid nature of these colonies could be demonstrated by the presence of CAI, HbF, and GPA in the cells by immunofluorescence labeling. In the absence of added Epo, only atypical erythroid colonies were obtained. Karyotypic analysis revealed two types of mitosis in unstimulated 24-hour blood cell cultures as well as in erythroid colonies (Table 4). Some had the Ph^1 chromosome as the only abnormality; the others had 48 to 64 chromosomes, including the Ph^1 chromosome; the representative karyotype of these cells was 53XX, +1, +3, +6, +10, +21, 22q−, t(9;22), 17p+.

The distinction between the morphologically abnormal erythroid colonies and the granulomonocytic colonies was difficult. In the first set of experiments, nearly all the morphologically presumed granulocytic colonies were in fact erythroid when studied with markers of differentiation. In a second set of experiments, we excluded the more dispersed colonies from plucking. Studies with MoAbs showed that 75% of the harvested colonies were indeed granulomonocytic, but some were erythroid; this could explain the finding of six hyperdiploid mitoses in granulomonocytic colonies. In the presence of Epo, some "morphologically normal" erythroid colonies of patient Val only had the Ph^1 chromosome. Finally, blood cells from patient 6 were grown in suspension for 2 months in the presence of a supernatant of the Mo-cell line.[39] More than 99% of the cells were granulocytic precursors at this time. Karyotypic analysis of these cells showed only the presence of a Ph^1 chromosome.

DISCUSSION

The nine cases presently described were found in a sample of 120 leukemic samples that could not be classified by

Table 4. Cytogenetics
Number of Metaphases Studied

	Number of Chromosomes																							
Material*	≤43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66
Case 6‡																								
B 24 h			7	2		2				4	6	3	5	3	1									
G M colonies (B)	2	5	4	5			1			1		1		1								1		
E colonies (B)		1	2	1					1	3	3	1	2	2	1									
2 month liquid culture (B)	2	1	1	2																				
Case 5‡																								
B 24 h														1	1	1	2	1	1	2	1	1		
E colonies (M)															1	1	3			3	5	3	1	1
E colonies (B)			1	3																				
G M colonies (M)			1	6																				
G M colonies (B)			3	9										1										
Case 9§																								
B 48 h	9	5	2°	2°																				
B 72 h	8	5	1°	5	2																			
E colonies	4	5°	9	1																				

Erythroid colonies were collected in cultures with Epo, GM colonies were grown with Mo-medium, PHA-LCM, or Mo-medium + Epo. °, 1 mitosis without marker; G M colonies, granulomonocytic colonies; E, erythroid; B, blood; M, bone marrow.

*M 48 h, B 24 h, B 48 h, B 72 h cultures of bone marrow for 48 hours, of blood for 24 hours, 48 hours, and 72 hours, respectively, without mitogens.

‡All metaphases with Ph^1 chromosome.

§All metaphases abnormal but not all identical.

morphological and cytochemical criteria. Their phenotype differs from all other cases (see Table 5)[40] by the constant expression of both CAI and the antigen identified by the FA6 MoAb. In addition, the morphology of the blast cells from these cases was similar in many ways and was characterized by extremely basophilic cytoplasm and a large nucleus with one or two large nucleoli. Ultrastructural investigation of these nine cases (to be reported) has shown ferritin molecules located in peculiar cytoplasmic granules in the absence of rhopheocytosis vesicles. We have recently detected cells with the same morphology and the same ferritin-containing granules in a highly enriched CFU-E fraction obtained from normal marrows.

Investigation with other erythroid markers listed in Table 3 led us to distinguish two main phenotypes that correspond to discrete stages of the normal erythroid differentiation (Fig 2):

(*a*) In patients 1 and 4, the phenotype is identical to that of an immature erythroblast. The main erythroid proteins are detected, including GPA identified by MoAbs.

(*b*) In six patients (patients 3 and 5 through 9), the phenotype is related to that of a late erythroid progenitor. Leukemic cells are identified only by the early markers (CAI, FA6, Gerbich and A antigens), which are expressed by the normal CFU-E.[13,14,31,41] HLA-DR antigen is not detected on the surface of these leukemic cells and has been demonstrated to be absent or expressed at low level by normal CFU-E.[41–44]

Leukemic cells from patient 2 have an intermediate phenotype between these two groups because in addition to the early markers, spectrin and GPA (detected by a polyclonal antibody) were present. This last result suggests that GPA is synthesized in this patient in an incompletely-*O*-glycosylated form.[45]

The erythroid origin of these leukemic cells was further sustained by the presence of the same karyotypic abnormalities in the blast cells and in the erythroid colonies obtained in three of these patients. It is noteworthy that in these three cases (patients 5, 6, and 9), cells composing the erythroid colonies have acquired the main erythroid proteins, such as Hb, whereas the blast cells were devoid of them. However, these colonies obtained with an unusually high plating efficiency were abnormal by their poor hemoglobinization and by their growth in the absence of added Epo. In patient 5, the karyotypic abnormalities added to the Ph^1 chromosome were present in the erythroid colonies but absent from the granulo-

Table 5. Correlation Between Immunological Phenotype and FAB Classification for Leukemia

Leukemic Subtype According to FAB Classification		Reactivity With Panel of Antibodies																
		CAI	MPO	vWF	20-6	My 10	My 9	My 7§	80 H 5	MO$_2$	B1	J5	B4	T11	AN 51	J15	FA6	R10 R18
Myeloblastic	MO*, M1	0	+	0	+	+	+	+/−	+/−	0	0	+/−	0	+/−	0	0	0	0
	M2, M3	0	+	0	0	0	+	+/−	+	0	0	0	0	+/−	0	0	0	0
Monocytic	M4	0	+	0	+	0	+	+/−	+	+	0	0	0	0	0	0	+	0
	M5	0	+	0	+	+/−	+/−	+/−	+	0	0	0	0	0	0	0	0	0
Megakaryoblastic	M7† mat[38]	0	0	+/−	+/−	0	0	0	0	0	0	0	0	+/−	+	+	+/−	0
	M7† immat[38]	0	0	0	+	+	+	?	+/−	0	0	0	0	0	0	+/−	0	0
Erythroblastic	M6 (eryth. component)	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+

Only the major cellular component is represented in this table. In some leukemic subtypes, other minor cellular components are frequently observed as megakaryoblasts in the M$_0$ and M$_1$ subtypes, myeloblasts in the M$_7$ and M$_6$ subtypes, or erythroblasts in the M$_7$ subtype. The lymphoid lineage-associated antigens identified by the J5 and T11 MoAb are expressed in some AML. This table is based on immunodiagnostic investigation of 310 cases of acute leukemia and blast crisis of CML.

*Cytochemistry for myeloperoxidase was negative by light microscopy; however, these cases exhibited rare small granules containing myeloperoxidase at the ultrastructural level and have been called MO. This leukemic subtype is often labeled by the 80H5 MoAb in contrast to the M$_1$ subtype.

†Revelation of platelet peroxidase was performed in these cases. The anti-vWF and the FA6-152 MoAb are less sensitive than the J15 and AN51 MoAbs to detect M7 mature leukemia.

§The antigen recognized by the My 7 MoAb is related to the cellular proliferation.

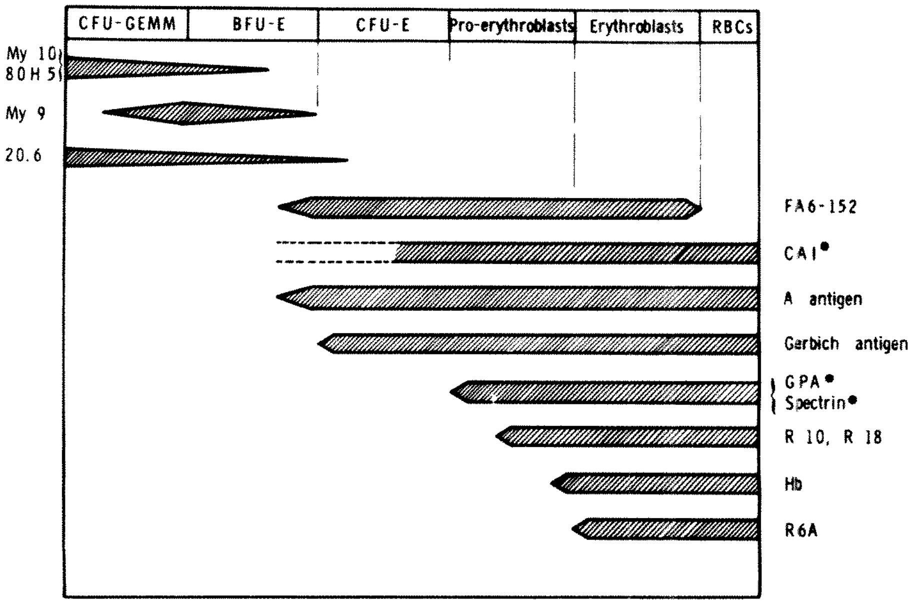

Fig 2. Pattern of the antigenic distribution-defined erythroid cell phenotypes during adult differentiation. The My7, Mo$_2$, B$_1$, J5, T$_{11}$, AN51, J15, and B$_4$ MoAbs-related antigens have not yet been detected during erythroid differentiation. *, antigens detected by polyclonal antibodies.

monocytic colonies with the exception of one ambiguous colony. The same result was obtained in patient 9, but we could not definitely separate the granulomonocytic from the erythroid colonies. However, in long-term suspension culture, the granulocytic lineage could be grown in the absence of erythroid precursors. In this case, the cells exhibited only the Ph^1 chromosome.

The phenotypes of these cases of erythroleukemia differ from those of the human erythroleukemic cell line K562, which expresses GPA and Hb[46-48]; however, K562 cells are devoid of CAI,[14] ABH antigens, and the antigen identified by FA6.[13] These nine cases are also apparently different from previously described cases of AML or ALL, in which some cells were doubly labeled by a polyclonal antibody against spectrin and the OKM$_1$ MoAb. From this result, it was suggested that leukemic myeloblasts may abnormally express erythroid-specific genes.[49] However, neither of these markers is truly lineage specific. Spectrin and spectrin-related proteins have been detected in non-erythroid cells,[50] including the lymphoid lineage.[51] The OKM$_1$ MoAb that recognizes the C$_3$b$_i$ receptor[52] binds to cells of the granulomonocytic lineage and to natural killer and some lymphoid cells.[53] In addition, OKM$_1$ MoAb has recently been shown to bind to normal CFU-E.[54]

Erythroleukemia appears to be uncommon among de novo leukemias (about 1% to 2% in this study), whereas it may be much more common in blast crisis of CML and in Down's syndrome.[7-11,55] Among the 120 studied patients, erythroleukemia represented four of 30 cases of CML blast crisis and three of ten cases of Down's syndrome leukemias.

In conclusion, erythroleukemia and erythroleukemic blast crisis of CML appear to be heterogenous. Most correspond to the CFU-E–like stage of the normal erythropoiesis. They may be an unique source of material with which to investigate the molecular mechanisms regulating human erythropoiesis.

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REFERENCES

1. Dameshek W: The Di Guglielmo syndrome revisited. Blood 34:567, 1969

2. Bennet JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukaemias. Br J Haematol 33:451, 1976

3. Bernheim A, Berger R, Daniel MT, Valensi F, Flandrin G: Malignant and reactive erythroblasts in erythroleukemia (M6) Cancer Genet Cytogenet 10:1, 1983

4. Fialkow PJ, Jacobson RJ, Papayannopoulou T: Chronic myelocytic leukemia. Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am J Med 63:125, 1977

5. Andersson LC, Gahmberg CG, Teerenhovi L, Vuopio P: Glycophorin A as a cell surface marker of early erythroid differentiation in acute leukemia. Int J Cancer 23:717, 1979

6. Andersson LC, Wegelius R, Borgstrom GH, Gahmberg CG: Change in cellular phenotype from lymphoid to erythroid in a case of AL. Scand J Haematol 23:115, 1980

7. Ekblom M, Borgstrom G, von Willebrand E, Gahmberg CG, Vuopio P, Andersson LC: Erythroid blast crisis in chronic myelogenous leukemia. Blood 62:591, 1983

8. Greaves MF, Sieff C, Edwards PAW: Monoclonal antiglycophorin as a probe for erythroleukemias. Blood 61:645, 1983

9. Liszka K, Majdic O, Bettelheim P, Knapp W: Glycophorin A expression in malignant hematopoiesis. Am J Hematol 15:219, 1983

10. Bettelheim P, Lutz D, Majdic O, Paietta E, Haas O, Linkesch W, Neumann E, Lechner K, Knapp W: Cell lineage heterogeneity in blast crisis of chronic myeloid leukaemia. Br J Haematol 59:395, 1985

11. Griffin JD, Todd RF, Ritz J, Nadler LM, Canellos GP, Rosenthal D, Gallivan M, Beveridge RP, Weinstein H, Karp D, Schlossman SF: Differentiation patterns in the blastic phase of chronic myeloid leukemia. Blood 61:85, 1983

12. Robinson J, Sieff C, Delia D, Edwards PAW, Greaves MF: Expression of cell-surface HLA-Dr, HLA-ABC and glycophorin during erythroid differentiation. Nature 289:68, 1981

13. Edelman P, Vinci G, Villeval JL, Vainchenker W, Henri A, Miglierina R, Rouger Ph, Reviron J, Breton-Gorius J, Sureau C, Edelman L: A monoclonal antibody against an erythrocyte ontogenic antigen identifies foetal and adult erythroid progenitors. Blood 67:56, 1986

14. Villeval JL, Testa U, Vinci G, Tonthat H, Bettaieb A, Titeux M, Cramer P, Edelman L, Rochant H, Breton-Gorius J, Vainchenker W: Carbonic anhydrase I is an early specific marker of normal human erythroid differentiation. Blood 66:1162, 1985

15. Boyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 21:51, 1968 (suppl 97)

16. Edwards PAW: Monoclonal antibodies that bind to the human erythrocyte-membrane glycoproteins glycophorin A and band 3. Biochem Soc Trans 8:334, 1980

17. Mannoni P, Janowska-Wieczorek A, Turner R, McGann L, Turc JM: Monoclonal antibodies against human granulocytes and myeloid differentiation antigens. Hum Immunol 5:309, 1982

18. Todd RF, Nadler LM, Schlossman SF: Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126:1435, 1981

19. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant cells. J Clin Invest 68:932, 1981

20. Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8:521, 1984

21. Cramer E, Pryzwansky KB, Villeval JL, Testa U, Breton-Gorius J: Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. Blood 65:423, 1985

22. McMichael AJ, Rust NA, Pilch JR, Sochynsky R, Morton J, Mason DY, Ruan C, Tobelem G, Caen J: Monoclonal antibody to human platelet glycoprotein. I. Immunological studies. Br J Haematol 43:501, 1982

23. Vainchenker W, Deschamps JF, Bastin JM, Guichard J, Titeux M, Breton-Gorius J, McMichael AJ: Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation. Immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in vivo cells from normal and leukemic patients. Blood 59:514, 1982

24. Kieffer N: Contribution à l'étude de la thrombospondine plaquettaire. Thèse, Université Paris, 1984

25. Tabilio A, Vainchenker W, van Haecke D, Vinci G, Guichard J, Henri A, Reyes F, Breton-Gorius J: Immunological characterization of the leukemic megakaryocytic line at light and electron microscopic levels. Leuk Res 8:769, 1984

26. Stashenko P, Nadler LM, Hardy R, Schlossman SF: Characterization of a human B lymphocyte-specific antigen. J Immunol 125:1678, 1980

27. Ritz J, Pesando JM, Notis-McConarty J, Lazaruz J, Schlossman SF: A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature 283:583, 1980

28. Verbi W, Greaves MF, Koubek K, Janossy G, Stein H, Kung P, Goldstein G: OKT 11 and OKT 11a: Monoclonal antibodies with pan T reactivity which block sheep erythrocyte receptors on T cells. Eur J Immunol 12:81, 1982

29. Charron DJ, McDevitt HO: Analysis of HLA-D region-associated molecules with monoclonal antibody. Proc Natl Acad Sci USA 76:6567, 1973

30. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH: Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133:157, 1984

31. Sieff CA, Caine G, Davis S: Cell surface antigens expressed on haemopoietic cells and the use of monoclonal antibody combinations to rapidly enrich for multipotent and committed progenitors. Br J Haematol 58:180, 1984 (abstr)

32. Henri A, Testa U, Tonthat H, Riou J, Titeux M, Vainchenker W, Feuillhade F, Galacteros F, Rochant H: Disappearance of Hb F and i antigen during the first year of life. Am J Hematol 9:161, 1980

33. Jackson CW: Cholinesterase as a possible marker of early cells of the megakaryocyte series. Blood 42:413, 1973

34. Aye MT, Seguin JA, McBurney JP: Erythroid and granulocytic colony growth in cultures supplemented with human serum lipoprotein. J Cell Physiol 99:233, 1979

35. Aye MT, Till JE, McCulloch EA: Interacting populations affecting proliferation of leukemic cells in culture. Blood 45:485, 1975

36. Iscn N: An international system for human cytogenetic nomenclature. Cytogenet Cell Genet 21:309, 1978

37. Dubé ID, Eaves CJ, Kalousek DK, Eaves AC: A method for obtaining high quality chromosome preparation from single hemopoietic colonies on a routine basis. Cancer Genet Cytogenet 4:157, 1981

38. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7). Ann Intern Med 103:460, 1985

39. Golde DW, Bersch N, Quan SG, Lusis AJ: Production of erythroid-potentiating activity by a human T-lymphoblast cell line. Proc Natl Acad Sci USA 77:593, 1980

40. Chan LC, Pegram SM, Greaves MF: Contribution of immunophenotype to the classification and differential diagnosis of acute leukaemia. Lancet 8427:475, 1985

41. Sieff C, Bicknell D, Caine G, Robinson J, Lam G, Greaves MF: Changes in cell surface antigen expression during hemopoietic differentiation. Blood 60:703, 1982

42. Blackblock HA, Katz F, Michalevitz R, Haylehurst GRP, Davies L, Prentice HG, Hoffbrand AV: A and B blood group antigen expression on mixed colony cells and erythroid precursors: Relevance for human allogeneic bone marrow transplantation. Br J Haematol 58:267, 1984

43. Linch DC, Nadler LM, Luther EA, Lipton JM: Discordant expression of human Ia-like antigens on hematopoietic progenitor cells. J Immunol 132:2324, 1984

44. Falkenburg JHF, Janssen J, Van der Vaart-Duin-Kerlen N, Veenhof WFJ, Blotkamp J, Goselink HM, Parlevliet J, Van Rood JJ: Polymorphic and monomorphic HLA-Dr determinants on human hematopoietic progenitor cells. Blood 63:1125, 1984

45. Gahmberg CG, Ekblom M, Andersson LC: Differentiation of human erythroid cells is associated with increased O-glycosylation of the major sialoglycoprotein, glycophorin A. Proc Natl Acad Sci USA 81:6752, 1984

46. Andersson LC, Nilsson K, Gahmberg CG: K 562: A human erythroleukemic cell line. Int J Cancer 23:143, 1979

47. Rutherford TR, Clegg JB, Weatherall DJ: K 562 human leukaemic cells synthesize embryonic haemoglobin in response to hemin. Nature 280:164, 1979

48. Villeval JL, Pelicci PG, Tabilio A, Titeux M, Henri A, Louache F, Thomopoulos P, Vainchenker W, Garbaz M, Rochant H, Breton-Gorius J, Edwards PAW, Testa U: Erythroid properties of K562 cells. Effect of hemin, butyrate and TPA induction. Exp Cell Res 146:423, 1983

49. Smith LJ, Curtis JE, Messner HA, Senn JS, Furthmayr H, McCulloch EA: Lineage infidelity in acute leukemia. Blood 61:1138, 1983

50. Lazarides E, Nelson WJ: Expression of spectrin in nonerythroid cells. Cell 31:505, 1982

51. Nelson WJ, Colaco CALS, Lazarides E: Involvement of spectrin in cell-surface receptor capping in lymphocytes. Proc Natl Acad Sci USA 80:1626, 1983

52. Sanchez-Madrid F, Nagy JA, Robbins E, Simon P, Springer TA: A human leukocyte differentiation antigen family with distinct α subunits and a common β subunit: The lymphocyte function-associated antigen (LFA-1), the C_3bi complement receptor (OKM_1, Mac-1) and the P150, 95 molecule. J Exp Med 158:1785, 1983

53. Reinhzerz EL, Moretta L, Roger M, Breard JM, Mingari MC, Cooper MD, Schossman SF: Human T lymphocyte subpopulations defined by Fc receptors and monoclonal antibodies. A comparison. J Exp Med 151:96, 1980

54. Falkenburg JH, Koning F, Duinkerken N, Fibbe WE, Voogt P, Jansen J: Expression of CDII, CDw15, and transferrin receptor antigens on human hematopoietic progenitor cells. Exp Hematol 14:90, 1986

55. Rosenthal S, Canellos GP, Gralnick HR: Erythroblastic transformation of chronic granulocytic leukemia. Am J Med 63:116, 1977

Hemoglobin Köln: Direct Analysis of the Gene Mutation by Synthetic DNA Probes

By J. Horst, R. Oehme, and E. Kohne

The molecular defect leading to Hb Köln has been analyzed by synthetic oligonucleotides. Thus, DNA of 19 nucleotides, in length corresponding to the normal and mutant β-globin gene sequences, were used to develop a direct assay for the β^k-gene that codes for this most common form of the unstable hemoglobins. The use of synthetic oligonucleotides established that the Hb Köln mutation is due to a G-to-A transition. The conditions described here should result in the determination of all Hb Köln genotypes with a high level of confidence.

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HEMOGLOBIN (Hb) Köln disease belongs to the group of nonspherocytic hemolytic anemias with a dominant inheritance pattern in which anemia is attributable to instability of the Hb molecule. The molecular pathology of this most common of the unstable Hbs was found to be the result of replacement of valine with methionine at β 98 (FG 5), causing increased oxygen affinity and instability, possibly because of hemedepletion.[1,2] The clinical features of patients with Hb Köln are those associated with hemolytic anemia. They may vary individually from mild to severe, and transfusion therapy might have to be started in childhood.

The underlying molecular defect in the chromosomal DNA leading to the amino acid substitution in Hb Köln has not directly been analyzed thus far, but it can be explained by a G-to-A transition caused by a point mutation. A direct detection of the mutation by virtue of the specificity of restriction enzymes was not possible because of the lack of suitable endonucleases. However, identification of the chromosomes that have passed the mutant gene on for three generations was possible by analyzing linked polymorphisms for DNA restriction sites within the β-globin gene cluster.[3] Although the results indicated the possible use of this method for prenatal diagnosis from DNA to amniotic fluid cells or chorion biopsy material, a diagnosis could only be expected in those cases where the haplotype constellations were informative. In all other cases prenatal diagnosis of the Hb Köln mutation has thus far been precluded because current Hb analysis is not available for identification of the β-anomaly from fetal blood with absolute certainty.

Here we report the detection of the Hb Köln hemoglobinopathy by direct analysis of the G-to-A transition mutation on the chromosomal DNA via synthetic oligonucleotide probes, thus establishing a method available for prenatal diagnosis of the disease.

MATERIAL AND METHODS

Oligonucleotides were labeled with adenosine 5'-γ-^{32}P-triphosphates (γ-^{32}P-ATP) (NEN, Boston; >7,000 Ci/mmol) by a kinase reaction[4] using T4 polynucleotide kinase (Boehringer, Mannheim). Labeled oligonucleotides were separated from unreacted γ-^{32}P-ATP by chromatography on Whatman (Maidstone, Kent, England) DE 52 cellulose.[5]

DNA samples of peripheral blood from normal and affected individuals were prepared as described.[6] Ten to 12 μg DNA were digested overnight with *Eco*RI (Bethesda Research Laboratories, Bethesda, Md) and additionally with *Hpa*I (Boehringer, Mannheim, Germany) in the buffer recommended by the commercial suppliers at 37 °C. The samples of the digested DNA were electrophoresed in 1% agarose. The gels were incubated overnight at room temperature in methanol (Epplen, personal communication) and dried for direct hybridization as described by Pirastu et al.[7]

After three and a half hours' hybridization the gels were washed in 6× SSC (1) four times for 30 minutes at room temperature, (2) overnight at 37 °C, and (3) finally for a short time at hybridization temperature (β^A-probes: Hβ19A, 55 °C) and at a 10 °C higher temperature respectively (β^k-probe: Hβ19k, 63 °C) as specified in figure legends and exposed to x-ray film (Kodak, XAR, Rochester, NY) with intensifying screens at −80 °C.

RESULTS

Mutational sequence and synthetic probes. The basis of the Hb Köln disease is the substitution of the valine residue at position 98 of the normal β^A-peptide chain by a methionine residue in the β^k-chain.[1] Sequencing analysis of the human β^A-globin gene has demonstrated that the codon for the valine residue at position 98 of the β^A-chain is GTG.[8] Since there is only one possible codon for methionine (ATG), it is very likely that the Hb Köln hemoglobinopathy is caused by a point mutation involving a G-to-A transition at the corresponding position in the gene (Fig 1A).

Recent investigations have shown that synthetic oligonucleotides exhibit an extremely specific hybridization behavior[5,13,14] and can therefore be used to detect single base-pair changes in genomic DNA.[15-18] The specific oligonucleotides complementary to the β^A- and β^k-genotypes of the β-globin gene (Fig 1B) were taken to establish a molecular method for distinction between the normal and the mutated genes.

Restriction fragments and genotype identification. Since it has been shown that restriction fragments one to three kilobases (kb) in size are optimal for the detection of single point mutations, combined digestions of the endonucleases *Eco*RI and *Hpa*I were selected from the several possibilities for the generation of 2.2-kb restriction frag-

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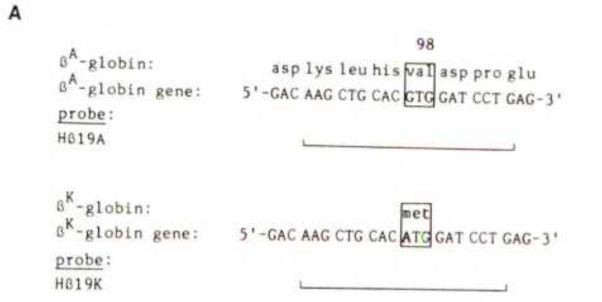
A
98
β^A-globin: asp lys leu his val asp pro glu
β^A-globin gene: 5'-GAC AAG CTG CAC GTG GAT CCT GAG-3'
probe:
Hβ19A
β^K-globin: met
β^K-globin gene: 5'-GAC AAG CTG CAC ATG GAT CCT GAG-3'
probe:
Hβ19K

B

Oligonucleotide	Mismatch(es) with globin genes					
	β^A	β^K	δ	$\psi\beta$	$^G\gamma/^A\gamma$	ε
Hβ19A	0	1	0	1	1	1
Hβ19K	1	0	1	2	2	2

Fig 1. A. Amino acid sequence and DNA sequence of the normal $\beta(\beta^A)$-globin gene[8] and Hb Köln $\beta(\beta^k)$-globin allele.[1,3] The connecting lines represent the DNA sequence of the synthetic oligonucleotides used in this study. B. Base-pair mismatch(es) in duplex of synthetic oligonucleotide probe and DNA of the β- and β-like globin genes.[8-12]

ments that contain the genomic DNA sequence of interest (Fig 2C). The specific hybridization conditions of the synthetic β^k-oligonucleotides to the respective genomic DNAs were established by test hybridization of the terminally labeled β^A- and β^k-probes to the cloned human β^A-globin gene (data not shown) as well as to chromosomal DNA of normal and Hb Köln-affected individuals (Fig 2A and B) of a German family.[3]

With the β^k synthetic oligonucleotide probe, neither the lanes containing the serially diluted cloned β^A-type/β-globin gene sequence (data not shown) nor the DNA from $\beta^A\beta^A$ genotype individuals showed hybridization signals (Fig 2B, lanes 4 to 6). Only DNA from the individuals with Hb Köln yielded hybridization signals in the 2.2-kb position. Thus, the synthetic β^k-oligonucleotide probe is capable of distinguishing $\beta^A\beta^A$ homozygotes from $\beta^A\beta^k$ heterozygotes.

DISCUSSION

We have adopted the recently described oligonucleotide mapping procedure for the analysis of single point mutations[5,14,15] to detect the Hb Köln mutations in chromosomal DNA of affected individuals. By means of synthetic β^A- and β^k-oligonucleotide probes it was possible to follow the segregation of the β^k-gene mutation through three generations of a family with Hb Köln–affected members.

The oligonucleotide analysis procedure has successfully been applied for diagnosis of some disorders like sickle cell anemia, β-thalassemias, and α_1-antitrypsin deficiency.[7,20,21] Here genotype analysis relies on the detection of normal homozygotes, heterozygotes, and defective homozygotes exhibiting the respective three sets of intense (+ +), intermediate (+ −), and missing (− −) band signals upon hybridization with oligonucleotides complementary to the normal (N) or the mutated (M) gene sequence: N + + + − − −/M − − + − + +.

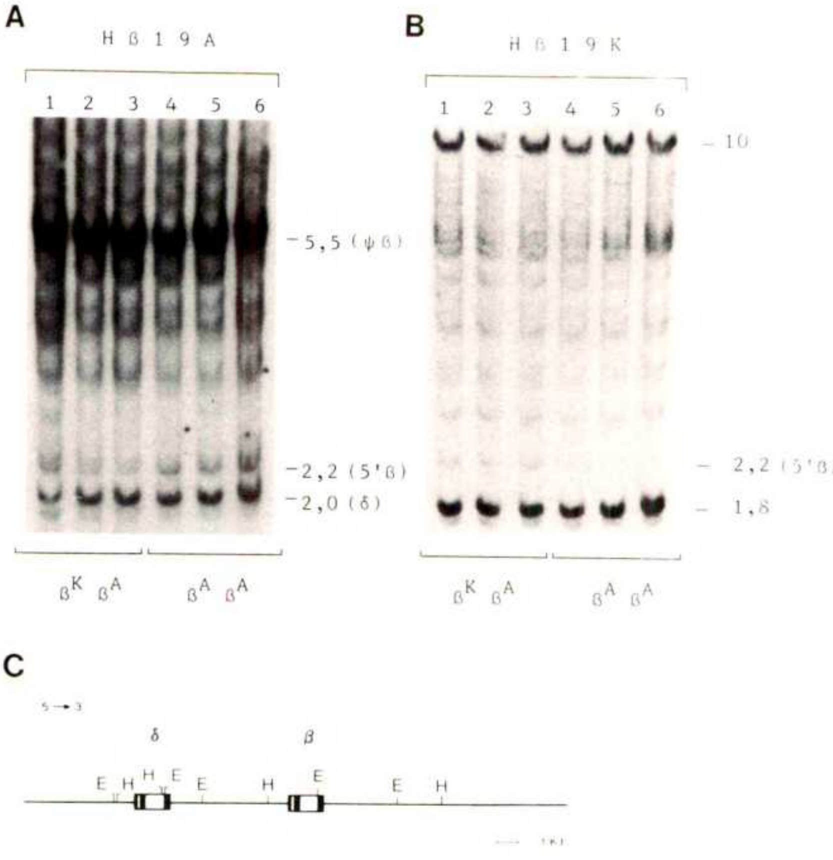

Fig. 2. Direct gel hybridizations of the human genomic DNAs of normal ($\beta^A\beta^A$) and Hb Köln–affected individuals ($\beta^k\beta^A$) with the 5'-^{32}P–end-labeled probes (A) Hβ19A (β^A specific) and (B) Hβ19k (β^k specific). DNAs were digested with the restriction enzymes *Eco*RI and *Hpa*I. The final wash of the two parallel gels (A and B) in 6 × SSC was carried out for (A) 2.5 minutes at 55 °C (hybridization temperature) and (B) three minutes at 63 °C (10 °C higher than the hybridization temperature). (C) Map of the *Eco*RI (E) and *Hpa*I restriction sites (H) in the β- and δ-globin gene region.[19]

In diseases with an autosomal dominant inheritance pattern, as in the Hb Köln disorder, normal homozygotes are differentiated from Hb Köln–affected ones by the first two sets of signals. Under controlled washing and hybridization conditions it can be seen that the probe specific for the mutated gene sequence yielded significant hybridization signals only with chromosomal DNAs from Hb Köln genotypes. The use of the synthetic oligonucleotide probes described here together with the enzymatic digests should therefore result in the determination of all Hb Köln genotypes with a high level of confidence. With regard to genetic counseling, this method could be used together with chorion biopsy or amniocentesis to provide prenatal diagnosis in families at risk.

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REFERENCES

1. Carrell RW, Lehmann H, Hutchison HE: Hemoglobin Köln (β 98 Valine-Methionine). An unstable protein causing inclusion body anemia. Nature 210:915, 1966
2. Winterbourn CC, Carrell RW: Studies of hemoglobin denaturation and Heinz body formation in the unstable hemoglobins. J Clin Invest 54:678, 1974
3. Horst J, Oehme R, Kleihauer E, Kohne E: Hemoglobin Köln: Analysis of linkage relationships between the mutant gene and polymorphic restriction sites in the β-globin gene cluster. Blut 48:213, 1984
4. Maniatis T, Fritsch EF, Sambrook J: Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, NY, 1982
5. Wallace RB, Schold M, Johnson MJ, Dembek P, Itakura K: Oligonucleotide directed mutagenesis of the human β-globin gene: A general method for producing specific point mutations in cloned DNA. Nucleic Acids Res 9:3647, 1981
6. Kan YM, Dozy AM: Polymorphism of DNA sequence adjacent to the human β-globin structural gene: Relationship to sickle mutation. Proc Natl Acad Sci USA 75:5631, 1978
7. Pirastu M, Kan YW, Cao A, Conner BJ, Teplitz RL, Wallace RB: Prenatal diagnosis of β-thalassemia. Detection of a single nucleotide mutation in man. N Engl J Med 309:284, 1983
8. Lawn RM, Efstratiadis A, O'Connell C, Maniatis T: The nucleotide sequence of the human β-globin gene. Cell 21:647, 1980
9. Spritz RA, de Riel JK, Forget BG, Weissman SM: Complete nucleotide sequence of the human δ-globin gene. Cell 21:639, 1980
10. Slightom JL, Blechl AE, Smithies O: Human fetal ${}^{G}\gamma$- and ${}^{A}\gamma$-globin genes: Complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. Cell 21:627, 1980
11. Baralle FE, Shoulder CC, Proudfoot NJ: The primary structure of the human ϵ-globin gene. Cell 21:621, 1980
12. Chang L-YE, Slightom JL: Isolation and nucleotide sequence analysis of the β-type globin pseudogene from human, gorilla and chimpanzee. J Mol Biol 180:767, 1984
13. Wallace RB, Shaffer J, Murphy RF, Bonner J, Hirose T, Itakura K: Hybridization of synthetic oligodeoxyribonucleotides to $\phi \times 174$ DNA: The effect of single base pair mismatch. Nucleic Acids Res 6:3543, 1979
14. Wallace RB, Johnson MJ, Hirose T, Miyake T, Kawashima EH, Itakura K: The use of synthetic oligonucleotides as hybridization probes. II. Hybridization of oligonucleotides of mixed sequence to rabbit β-globin DNA. Nucleic Acids Res 9:879, 1981
15. Suggs SV, Hirose T, Miyake T, Kawashima EH, Johnson MJ, Itakura K, Wallace RB: Use of synthetic oligodeoxyribonucleotides for the isolation of specific cloned DNA sequences, in Brown DD (ed): Developmental Biology Using Purified Genes. Orlando, Fla, Academic, 1981, p 683
16. Conner BJ, Reyes AA, Morin C, Itakura K, Teplitz RL, Wallace RB: Detection of sickle cell β^{S}-globin allele by hybridization with synthetic oligonucleotides. Proc Natl Acad Sci USA 80:278, 1983
17. Pirastu M, Kan YW, Galanello R, Cao A: Multiple mutations produce $\delta\beta^{o}$ thalassemia in Sardinia. Science 223:929, 1984
18. Kidd VJ, Wallace RB, Itakura K, Woo SL: α_1-Antitrypsin deficiency detection by direct analysis of the mutation in the gene. Nature 304:230, 1983
19. Fritsch EF, Lawn RM, Maniatis T: Molecular cloning and characterization of the human β-like globin gene cluster. Cell 19:959, 1980
20. Studencki AB, Conner BJ, Impraim CC, Teplitz RL, Wallace RB: Discrimination among the human β^{A}, β^{S}, and β^{C}-globin genes using allele-specific oligonucleotide hybridization probes. Am J Hum Genet 37:42, 1985
21. Orkin SH, Markham AF, Kazazian HH Jr: Direct detection of the common Mediterranean β-thalassemia gene with synthetic DNA probes: An alternative approach for prenatal diagnosis. J Clin Invest 71:775, 1983

CONCISE REPORT

Autocrine Secretion of GM-CSF in Acute Myeloblastic Leukemia

By Diane C. Young and James D. Griffin

Three cases of acute myeloblastic leukemia (AML) were identified in which clonogenic cells proliferated autonomously in vitro. Cells from two of these cases were found to secrete a colony-stimulating factor (CSF) that was immunologically and molecularly related to GM-CSF. Growth of AML-CFU could be blocked by the addition of a neutralizing antiserum to GM-CSF. Northern blot hybridization of leukemic cell mRNA with a cDNA probe for the GM-CSF gene revealed a 1-kb message identical in size to the normal GM-CSF message in stimulated T cells. No GM-CSF message was detected in the third case. These results indicate that constituitive expression of the GM-CSF gene, apparently by leukemic cells, can result in autonomous in vitro proliferation of AML-CFU in some cases of AML.

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ACUTE MYELOBLASTIC LEUKEMIA (AML) is a highly lethal malignancy characterized by abnormal proliferation and differentiation of myeloid progenitor cells. The mechanisms that have allowed AML cells to escape from normal regulatory events are unknown. When AML cells are cultured in semisolid media, only a small subset of cells is capable of in vitro proliferation.[1] These leukemic colony-forming cells (AML-CFU) share several features with normal myeloid progenitor cells, including a high thymidine suicide index,[2] self-renewal potential,[3] and the ability to undergo limited, although abnormal, differentiation to nonproliferative cells.[4] As with normal granulocyte-monocyte progenitor cells, the proliferation of AML-CFU generally requires the addition of colony-stimulating factors (CSFs).[1,3] We examined AML-CFU from 15 patients with AML for proliferation in the absence of added CSFs and found that in three cases, colony formation occurred autonomously in agar. Medium conditioned by the leukemic cells from two of these cases was found to contain a factor that could stimulate proliferation of normal bone marrow granulocyte, monocyte, and eosinophil colony growth, as well as proliferation of AML-CFU from other AML patients. This report describes the immunological and molecular characterization of the factor secreted by the leukemic cells in these cases.

MATERIALS AND METHODS

Leukemic cells from pretreatment bone marrow aspirate samples were cryopreserved until use. The diagnosis of AML in each case was established by morphology, cytochemistry, and surface antigen analysis. All samples contained more than 95% blasts and less than 1% T lymphocytes (detected by immunofluorescence staining with anti-T3). The leukemic cells lacked expression of T cell markers T1, T3, or T11. In all experiments, residual T cells were further depleted by rosetting with sheep erythrocytes, and monocytes were depleted by plastic adherence.

Secretion of CSF by leukemic cells was determined by assaying medium conditioned by leukemic cells for the presence of colony-stimulating activity for normal bone marrow CFU-GM. Conditioned medium (CM) was prepared by culturing AML cells at 1 × 10^6/mL in RPMI 1640 containing 10% fetal bovine serum for 48 hours. Phytohemagglutinin (PHA)-stimulated (2 μg/mL) normal leukocytes were similarly cultured to prepare CM as a positive control. CM was then incorporated at various dilutions into the underlayer of a double-layer agar assay system.[4] The agar overlayer contained 10^5/mL light density normal bone marrow mononuclear cells. On day 14, colonies were stained in situ for alpha-naphthyl acetate esterase (monocytes), chloroacetate esterase (granulocytes), and Luxol fast blue (eosinophils).[5]

AML-CFU were assayed as described[4] in a double-layer agar culture system (1 to 5 × 10^4 cells per culture). Where appropriate, purified recombinant GM-CSF (r-GM-CSF)[6] or sheep anti-GM-CSF antiserum (both provided by Dr Steven Clark, Genetics Institute, Cambridge, Mass) were added to the underlayer. The GM-CSF had been purified to a single band from medium conditioned by COS cells transfected with a vector containing the GM-CSF gene.[6] The anti–GM-CSF sheep serum had been prepared against purified recombinant GM-CSF and reacted with a single broad band (apparent molecular weight 19 to 26 kd) by Western blot of the r-GM-CSF preparation used in this study. Clusters of more than eight leukemic blasts were counted on day 7. Preimmune sheep serum at the same dilution had no effect on colony growth.

Total cellular RNA (10 μg) was isolated as described.[7] Samples were fractionated on a 1.2% agarose gel with 6% formaldehyde and transferred to a nylon membrane (Gene Screen Plus, New England Nuclear, Boston). The membrane was hybridized to the *Aha*III-*Eco*RI fragment of the human GM-CSF cDNA labeled to a specific activity of 10^9 cpm/μg using oligonucleotide primers and ^{32}P-dCTP, as described.[8] Hybridization was performed at 60 °C in a solution containing 1 mol/L NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100 μg/mL salmon sperm DNA, and 5 × 10^5 cpm/mL labeled probe. The membrane was washed with 2× SSC/1% SDS for one hour at 60 °C and 0.1XSSC at room temperature for one hour. In some experiments, the transfer membranes were washed free of GM-CSF probe with boiling 0.1× SSC/0.1% SDS and rehybridized with a cDNA probe for the constant region of the alpha chain of the T cell antigen receptor, Ti,[9] supplied by Dr Ellis Reinherz (Dana-Farber Cancer Institute).

RESULTS

Medium conditioned by leukemic cells for 48 hours was tested for CSF activity and compared with a standard source of medium conditioned by lectin-stimulated T cells (Table

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Table 1. Secretion of CSF by Leukemic Cells

Case	Conditioned Medium (%)	D.14 CFU-GM/10^5 Marrow Cells Granulocyte	G/M or Monocyte	Eosinophil
1	10	44	28	9
	1	41	31	8
	0.1	42	32	8
	0.01	33	21	3
2	10	37	31	6
	1	21	26	2
	0.1	3	7	0
3	10	0	0	0
	1	0	0	0
	0.1	0	0	0
PHA-LCM	10	35	37	9
	1	6	12	1
	0.1	1	1	0
	0	0	0	0

Medium conditioned by culture with 1 × 10^6 AML cells per milliliter for 48 hours was assayed for CSF activity using normal marrow CFU-GM as target cells. At day 14, colonies were stained in situ for chloroacetate esterase, alpha naphthyl acetate esterase, and Luxol fast blue to determine colony composition. PHA-LCM, phytohemagglutinin-stimulated normal leukocytes.

1). A CSF activity for normal marrow granulocyte, monocyte, and eosinophil colony-forming cells (CFU-GM) was found in media from two of the leukemic samples. The titer of CSF activity in medium from case 1 was tenfold to 100-fold higher than in medium from case 2 or PHA-stimulated T cells. Promotion of eosinophil colony growth suggested a functional similarity with GM-CSF, as human G-CSF and CSF-1 do not promote eosinophil colony formation.[10] Medium from case 3 or from unstimulated T cells, monocytes, or normal marrow mononuclear cells did not contain detectable CSF activity.

The effects of adding a monospecific neutralizing antiserum to agar cultures of AML-CFU from these cases was tested (Table 2). Anti–GM-CSF antibody markedly diminished autonomous colony formation in cases 1 and 2 but had no detectable effect in case 3. The inhibitory effects of the antiserum could be abrogated by adding an excess of GM-CSF. The effects of adding purified recombinant GM-CSF[6] alone were also tested. In case 1, addition of GM-CSF did not increase AML-CFU number, but did increase colony size, whereas in cases 2 and 3, both colony size and number were increased.

Because the cells of cases 1 and 2 were apparently secreting a CSF functionally and immunologically related to T cell GM-CSF, further experiments were undertaken to exclude the possibility that contaminating T cells were secreting GM-CSF despite the absence of morphologically or immunologically identifiable T cells. Immunofluorescence analysis using anti-T11 as a pan-T cell marker revealed less than 0.1% T11+ cells in cases 1 through 3. Leukemic cells from cases 1 and 2 were also positively selected by fluorescence-activated cell sorting after staining with myeloid cell surface antigen–specific monoclonal antibodies anti-MY7 or anti-MY9.[12] In each case, medium conditioned by positively selected leukemic cells (>99% MY7+ or MY9+) contained titers of CSF as high as found in medium conditioned by unsorted cells. In other experiments not shown, conditioned media from cases 1 and 2 were assayed for the presence of two other T cell lymphokines: gamma-interferon (γ-IFN) and interleukin 2 (IL 2). The amounts of these T lymphokines was below the level of detection (less than 5 U/mL of γ-IFN and 0.1 U/mL IL 2). Sorting experiments were also done with the antimonocyte antibody MY4[11] in case 1 (case 1 had 5% MY4+ cells; case 2, 0%) to determine if only leukemic "monocytes" could secrete CSF. High titers of CSF were found in both MY4+ and MY4− fractions.

The nature of the CSF secreted by these leukemic cells was further investigated by Northern blot analysis[7] of leukemic cell mRNA using a cDNA probe for GM-CSF.[6] A 1.0-kb message was detected in cases 1 and 2, but no message was detected in case 3 (Figs 1A and 2). The message was identical in size to that found in PHA- or phorbol myristate acetate–stimulated normal T cells. In data not shown, this message was shown to be undetectable in unstimulated normal T cells, normal monocytes, or T cell–depleted bone marrow mononuclear cells. Rehybridization of the AML blots with a probe for the alpha chain of the T cell receptor[9] showed absence of this T cell–specific message (Fig 1B), further suggesting that T cells were not the source of GM-CSF in these leukemic specimens.

DISCUSSION

The results described here suggest that constituitive secretion of GM-CSF in human AML can promote autonomous proliferation of leukemic cells in vitro. In normal cells, the GM-CSF gene is expressed by activated T cells, but not by resting T cells or other hematopoietic cells.[6,12] In particular, normal monocytes and T-depleted marrow cells have not been shown to express the GM-CSF gene.[12] We therefore attempted to determine if the GM-CSF in the AML samples studied here was from T cells or leukemic cells. The samples contained less than 0.1% T cells by immunofluorescence, and rehybridization of the Northern blots with a probe specific for a "marker" T cell message (the alpha chain of the T cell

Table 2. Autonomous Proliferation of AML-CFU

Case No. (Sex, Age, FAB Classification)	AML-CFU/10^4 Cells Medium Control	Anti–GM-CSF (1/100)	Anti–GM-CSF +r-GM-CSF	r-GM CSF (0.1 μg/mL)
1. F, 4 yr, FAB M1	1,090 ± 110	80 ± 9	620 ± 97	1,080 ± 131
2. M, 37 yr, FAB M1	81 ± 29	17 ± 15	226 ± 6	299 ± 19
3. M, 73 yr, FAB M4	255 ± 40	286 ± 24	280 ± 24	387 ± 50

The French-American-British (FAB) classification was determined as described.[11] Anti–GM-CSF serum (sheep) was incorporated into the culture system at a final dilution of 1:100.

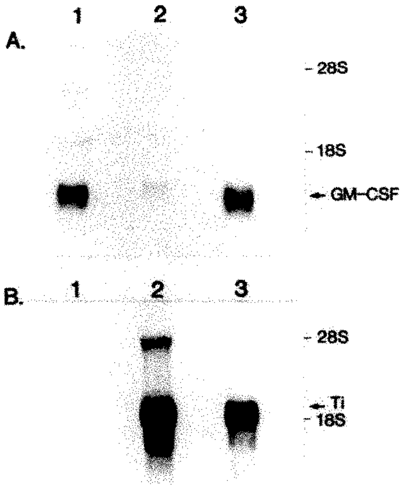

Fig 1. Northern blot analysis of T lymphocytes and case 1 AML mRNA. Total cellular RNA was fractional on an agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled cDNA probe for GM-CSF (A). The same blot was then washed and rehybridized with a cDNA probe for the alpha chain of the T cell antigen receptor Ti (B). Lane 1, AML case 1; lane 2, PHA-stimulated T cells; lane 3, PMA-stimulated T cells.

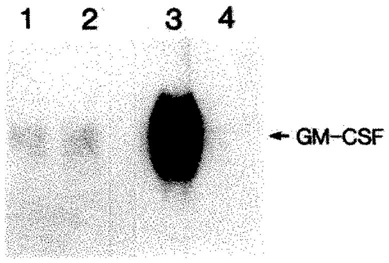

Fig 2. Northern blot analysis of GM-CSF mRNA from AML cases 1, 2, and 3. Lane 1, normal T lymphocytes stimulated with phorbol myristate acetate; lane 2, case 1; lane 3, case 2; lane 4, case 3.

antigen receptor) revealed no detectable T cell RNA. Further, other T cell lymphokines (γ-IFN and IL 2) were absent. These results favor the conclusion that the leukemic cells themselves are the source of GM-CSF, but the possibility that a small subset of non-leukemic cells is selectively secreting GM-CSF can not be excluded by these methods.

Although GM-CSF transcripts were not detected in normal marrow mononuclear cells, myeloid progenitor cells are a small fraction of all marrow cells, and it is not possible to exclude the possibility that normal progenitor cells also express the GM-CSF gene. This is unlikely, however, since purified normal myeloid progenitor cells neither proliferate autonomously nor secrete detectable CSF (J.D.G., unpublished observations, 1986). Studies on the regulation of the GM-CSF gene in AML and in T cells may help to clarify some of these issues. It is noteworthy that the amount of biologically active GM-CSF secreted by cases 1 and 2 did not correlate with the amount of GM-CSF mRNA. There are many possible explanations, including the production of an inactive, unstable, or nonsecreted CSF, or an mRNA that is not readily translated.

It is unlikely that "autocrine" secretion of a CSF could be the sole cause of AML, since a block in differentiation is also necessary to cause a disorder such as AML. However, because AML cells frequently use GM-CSF to proliferate in vitro,[13] it is possible that production of this factor by some cases of AML contributes to the overwhelming cellular proliferation characteristic of AML. Further, these results support hypotheses based on murine myeloid cell lines[14,15] and avian marrow cells[16,17] that abnormalities of CSF genes may contribute to the pathogenesis and abnormal proliferation of leukemias.

The cause of the constituitive expression of the GM-CSF gene in the two cases of AML reported here will require further investigation. Neither case 1 nor case 2 had morphologically detectable abnormalities of the long arm of chromosome 5, the location of the GM-CSF gene. Southern blot analysis of DNA from cases 1 and 2 cut with restriction endonucleases *Eco*R1, *Hind*III, *Bam*H1, and *BglI* has not revealed GM-CSF gene rearrangements using both a cDNA probe and a genomic probe (pCH 5.2)[18] and further does not suggest gene amplification. The constituitive activation of the GM-CSF gene in AML may be due to mutations in regulatory sequences for the gene not detected by the probes used in this study. Finally, autonomous proliferation of AML-CFU in case 3 was not due to GM-CSF expression, suggesting that other mechanisms exist to bypass the CSF requirement.

REFERENCES

1. Moore MAS, Williams N, Metcalf D: In vitro colony formation by normal and leukemic human hematopoietic cells: Characterization of the colony-forming cells. J Natl Cancer Inst 50:603, 1973

2. Minden MD, Till JE, McCulloch EA: Proliferative state of blast cell progenitors in acute myeloblastic leukemia (AML). Blood 52:592, 1978

3. Buick RN, Minden MD, McCulloch EA: Self-renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. Blood 54:95, 1979

4. Griffin JD, Larcom P, Schlossman SF: Use of surface markers to identify a subset of acute myelomonocytic leukemia cells with progenitor cell properties. Blood 64:693, 1984

5. Phillips PG, Chikkappa G, Brinson PS: A triple stain technique to evaluate monocyte, neutrophil and eosinophil proliferation in soft agar cultures. Exp Hematol 11:10, 1983

6. Wong GG, Witek JS, Temple PA, Wilkins KM, Leary AC, Luxemburg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985

7. Maniatis T, Fritsch EF, Sambrook J: Molecular cloning—a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1982

8. Feinberg AP, Vogelstein B: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6, 1983

9. Royer HD, Ramarli D, Acuto O, Campen TJ, Reinherz EL: Genes encoding the T cell receptor alpha and beta subunits are transcribed in an ordered manner during intrathymic ontogeny. Proc Natl Acad Sci USA 82:5510, 1985

10. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang

EA, Wong GG, Clark SC: Human recombinant granulocyte-macrophage colony stimulating factor: A multi-lineage hematopoietin. Science 230:1171, 1985

11. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, Sultan C: Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med 103:620, 1985

12. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 68:932, 1981

13. Nimer SD, Chan J, Slamon DJ, Golde DW, Gasson JD: Expression of human granulocyte-macrophage colony stimulating factor (GM-CSF). Blood 66:158a, 1985

14. Griffin JD, Young D, Herrmann F, Wiper D, Wagner K, Sabbath KD: Effects of recombinant GM-CSF on proliferation of clonogenic cells in acute myeloblastic leukemia. Blood 67:1448, 1986

15. Lang RA, Metcalf D, Gough NM, Dunn AR, Gonda TJ: Expression of a hemopoietic growth factor cDNA in a factor-dependent cell line results in autonomous growth and tumorigenicity. Cell 43:531, 1985

16. Ymer S, Tucker QJ, Sanderson CJ, Hapel AJ, Campbell HD, Young IG: Constituitive synthesis of interleukin-3 by leukemia cell line WEHI-3B is due to retroviral insertion near the gene. Nature 317:255, 1985

17. Adkins B, Leutz A, Graf T: Autocrine growth induced by *src*-related oncogenes in transformed chicken myeloid cells. Cell 39:439, 1984

18. Graf T, Weizsaeker FV, Grieser S, Coll J, Stehelin D, Patschinsky T, Bister K, Bechade C, Calothy G, Leutz A: *v-mil* induces autocrine growth and enhanced tumorigenicity in *v-myc*-transformed avian macrophages. Cell 45:357, 1986

19. Huebner K, Isobe M, Croce CM, Golde DW, Kaufman SE, Gasson JC: The human gene encoding GM-CSF is at 5q21-q32, the chromosome region deleted in the 5q− anomaly. Science 230:1282, 1985

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Blood, Vol 68, No 5 (November), 1986: 1182–1183

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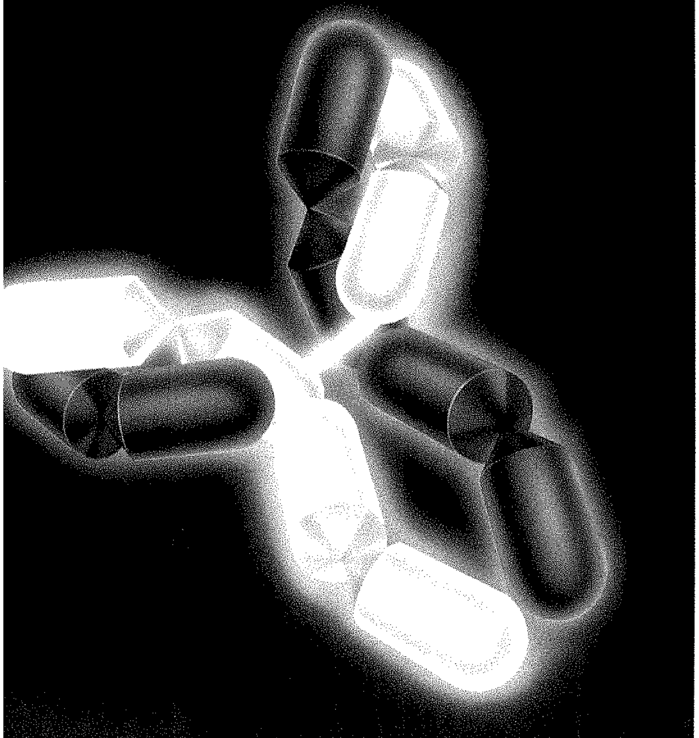

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Wedgwood, Ralph J.,** ***et al.**, (1986):** *Intravenous Immunoglobulin Home Treatment for Patients with Primary Immunodeficiency Diseases.* **Lancet,** (March 15), pp. 610-611.

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CONTRAINDICATIONS

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WARNINGS

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Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

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Pregnancy Category C

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The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

REFERENCES

1. Unpublished data in the files of Travenol Laboratories, Inc.
2. Waldmann TA, Storber W: Metabolism of immunoglobulins. *Prog Allergy 13*: 1-110, 1969
3. Morell A, Riesen W: Structure, function and catabolism of immunoglobulins in *Immunohemotherapy*. Nydegger UE (ed), London, Academic Press, 1981, pp 17-26
4. Stiehm ER: Standard and special human immune serum globulins as therapeutic agents. *Pediatrics* 63:301-319, 1979
5. Buckley RH: Immunoglobulin replacement therapy: indications and contraindications for use and variable IgG levels achieved in *Immunoglobulins: Characteristics and Use of Intravenous Preparations*. Alving BM, Finlayson JS (eds), Washington, DC, U.S. Department of Health and Human Services, 1979, pp3-8
6. Burks AW, Sampson HA, Buckley RH: Anaphylactic reactions following gammaglobulin administration in patients with hypogammaglobulinemia: detection of IgE antibodies to IgA. Submitted for publication.

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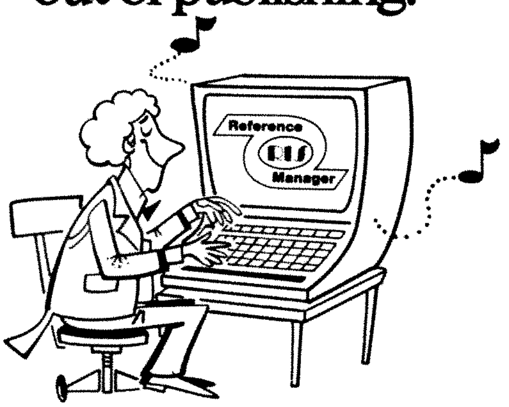

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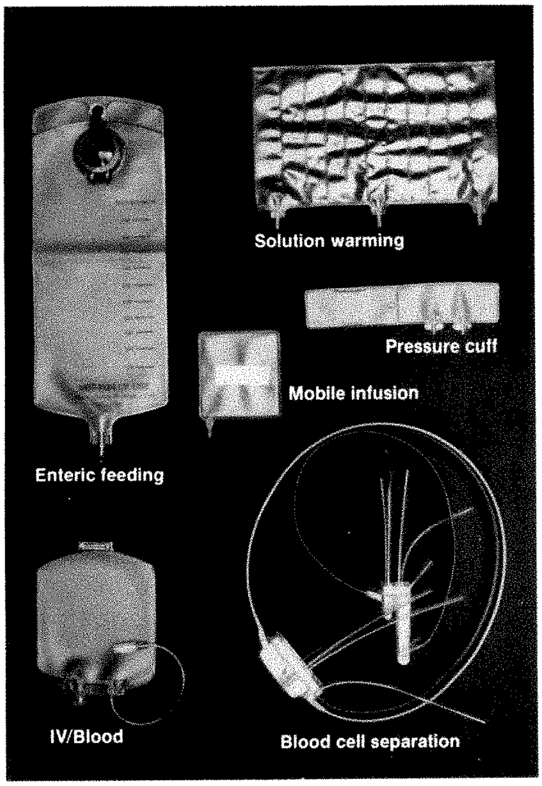

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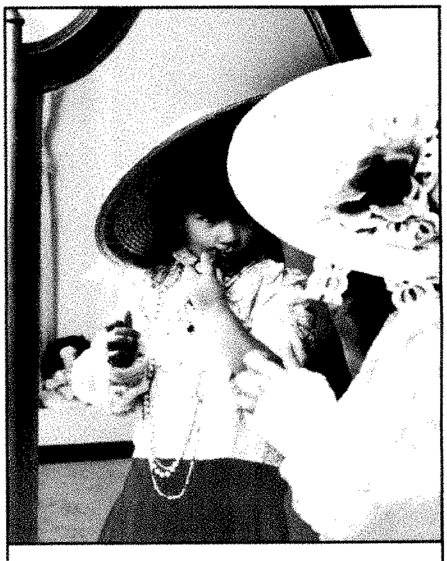

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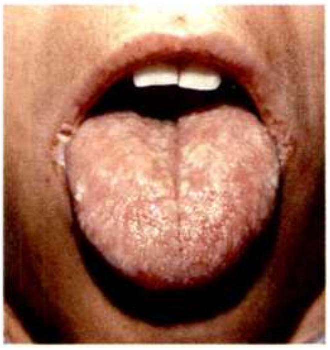
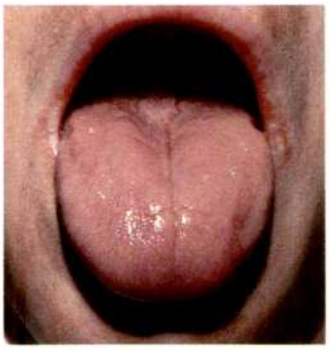


Before treatment.[1]



After treatment with MYCELEX Troche.[1]

High clinical cure* rates.

A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

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Patients attain a high degree of relief at the end of therapy, as evidenced by the dramatic decrease in severity of glossitis, patches, perleche, pain, and other clinical signs and symptoms of thrush.[4]

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Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole…in the treatment of thrush in any patient population…is both effective and safe."[2†]

*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

Mycelex® Troche

(clotrimazole) 10 mg

The only troche for oropharyngeal candidiasis.

MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

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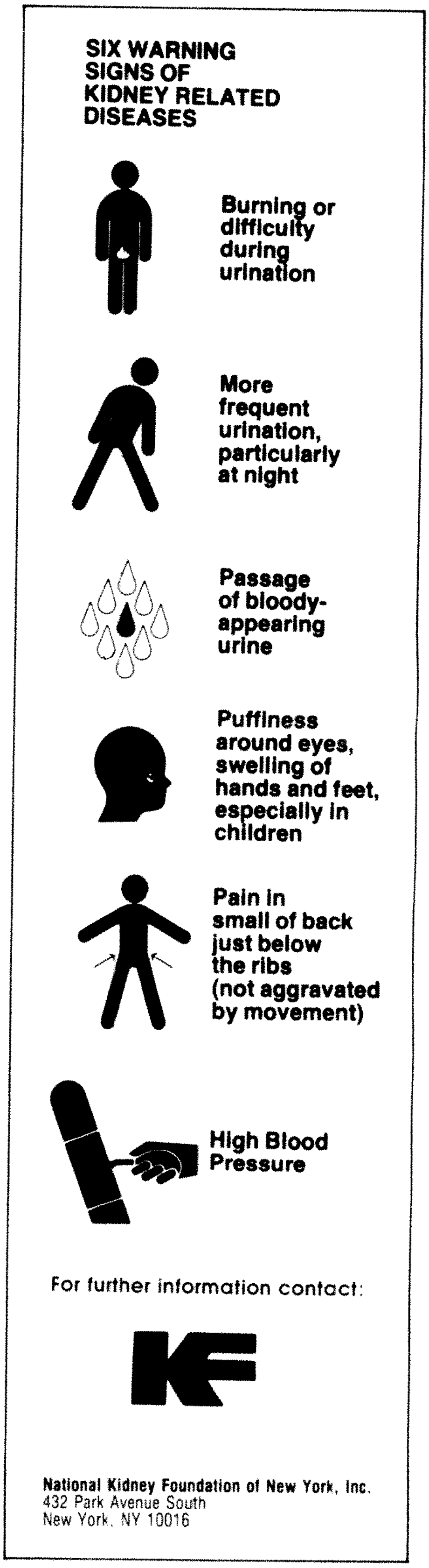

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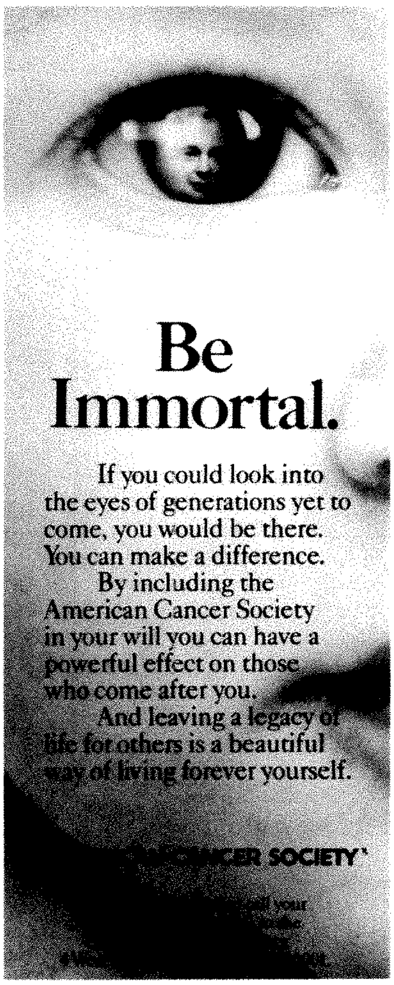

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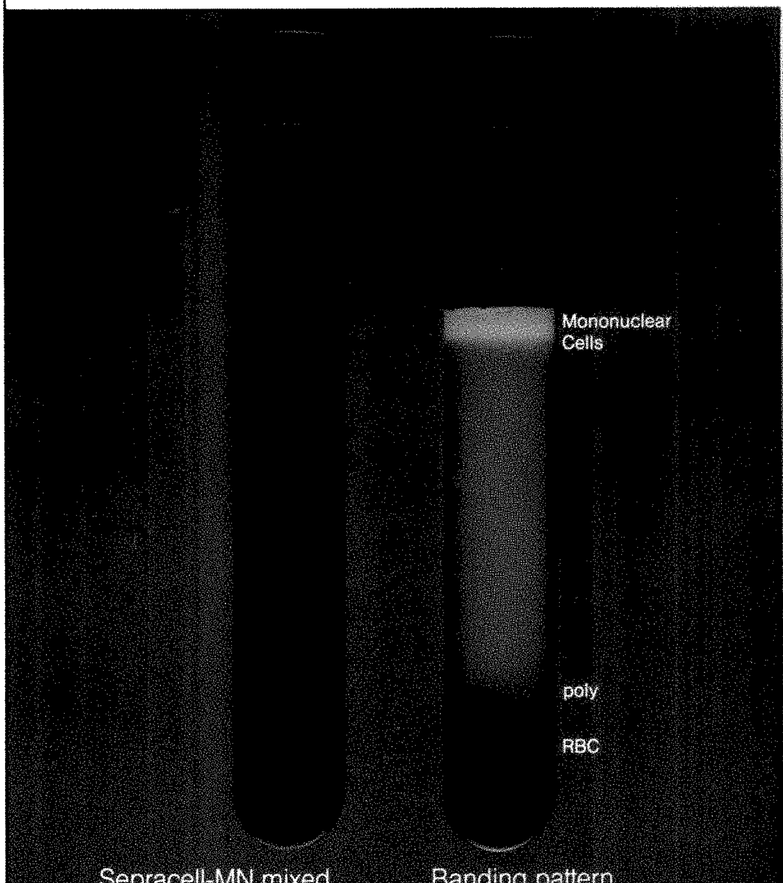

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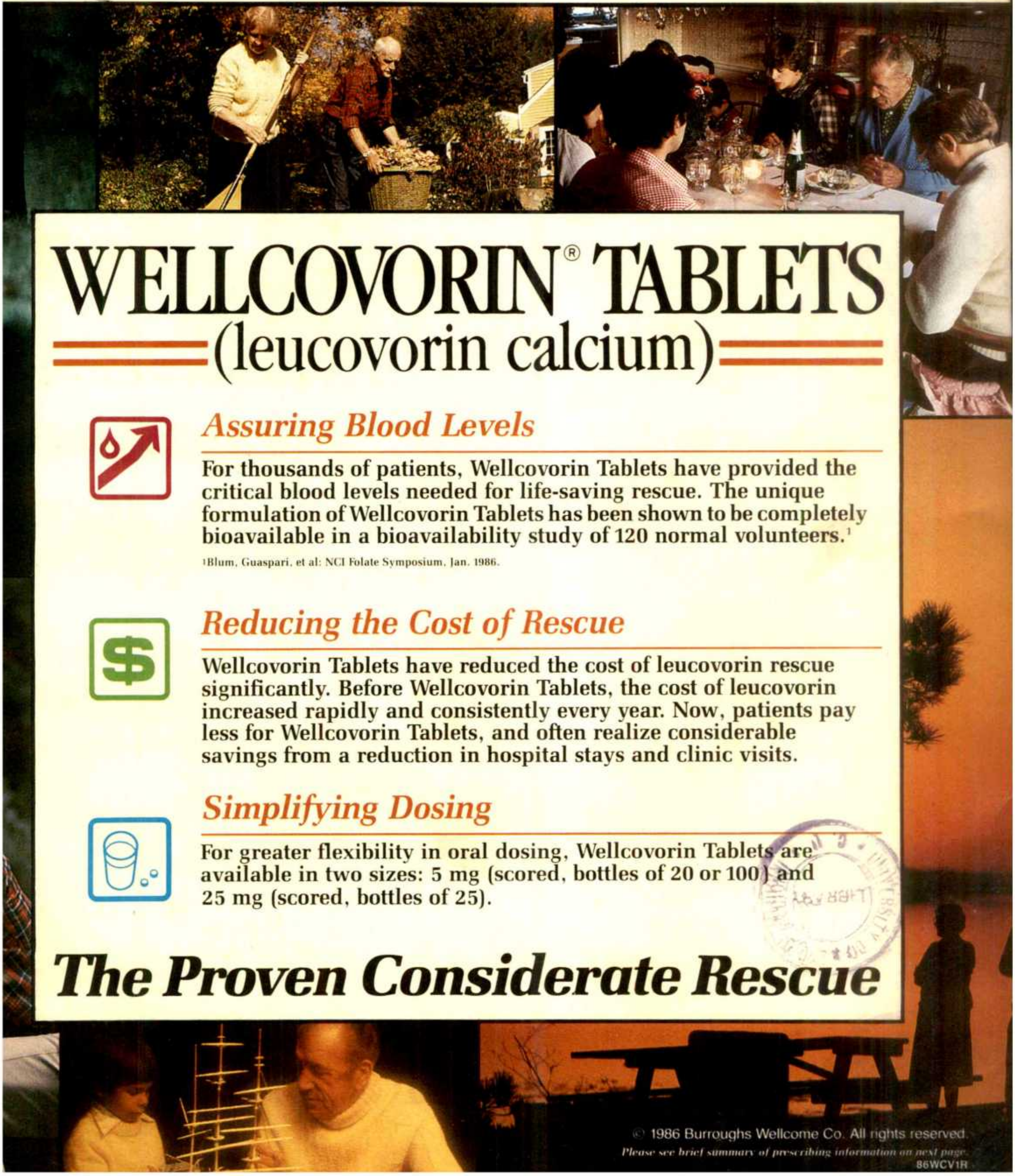

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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Please see brief summary of prescribing information on next page.
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Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al:* High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al:* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis. 135*(3): 493-496, 1977.

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"If everyone over 50 had checkups for colorectal cancer, the cure rate could be as high as 75%," says Dr. LaSalle D. Leffall, Jr., past president, American Cancer Society. "You can't cure it if you don't know you have it." But if it's detected early, the cure rate for colorectal cancer is very high. Your doctor can perform the digital and proctoscopic exams, and you take care of the simple stool blood test at home.

Since men and women are equally affected by this disease, we urge everyone over 50 to get regular checkups.

The warning signs for colorectal cancer are a change in bowel habits and blood in the stool.

People with a family history of colon or rectal cancer or ulcerative colitis are at higher risk and are urged to be doubly cautious.

Checkup Guidelines for men and women over 50 without symptoms:
- digital exam annually
- stool blood test annually
- procto exam every 3 to 5 years after 2 negative tests 1 year apart.

No one faces cancer alone.

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The Journal of
The American Society of Hematology

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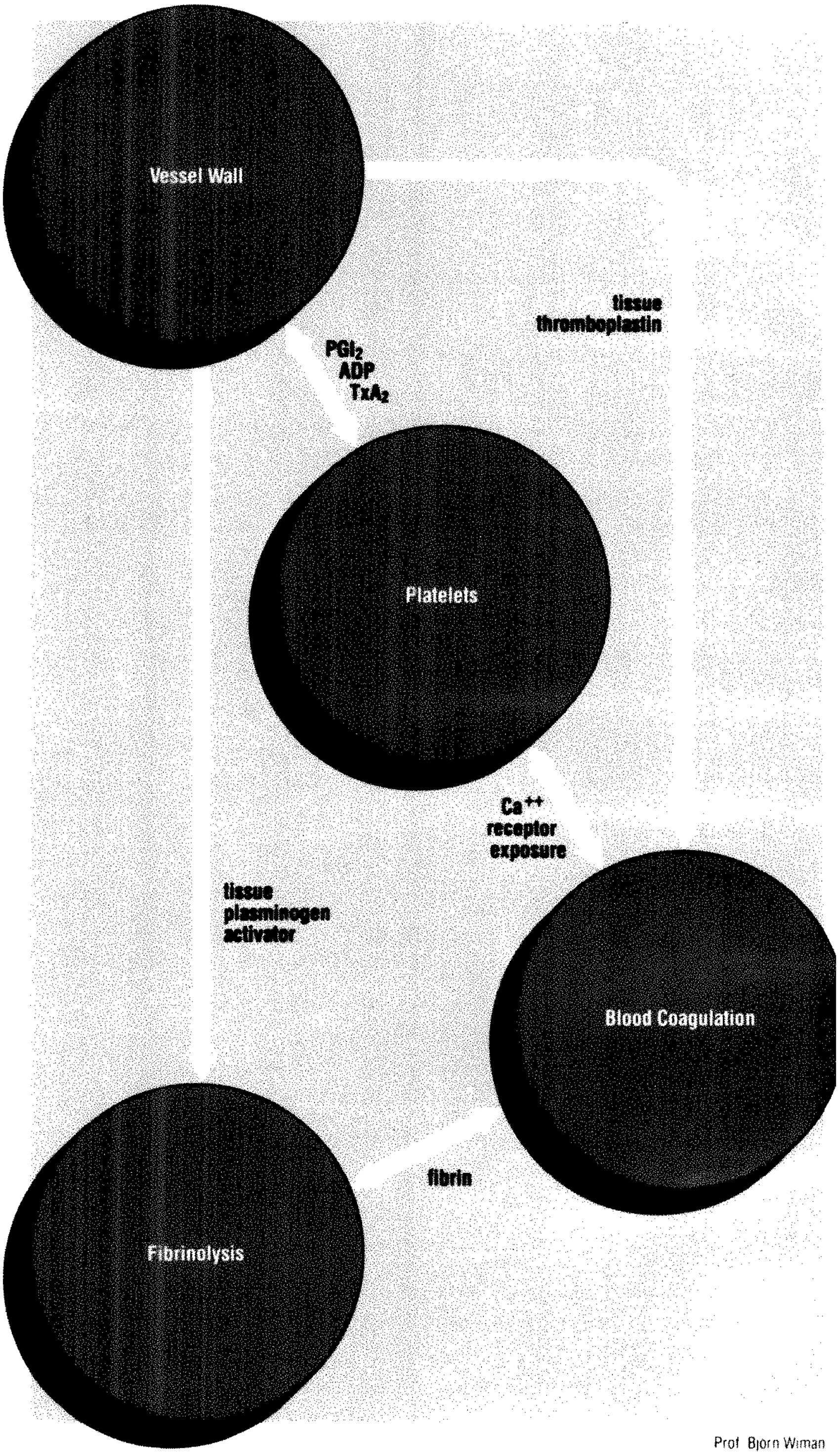

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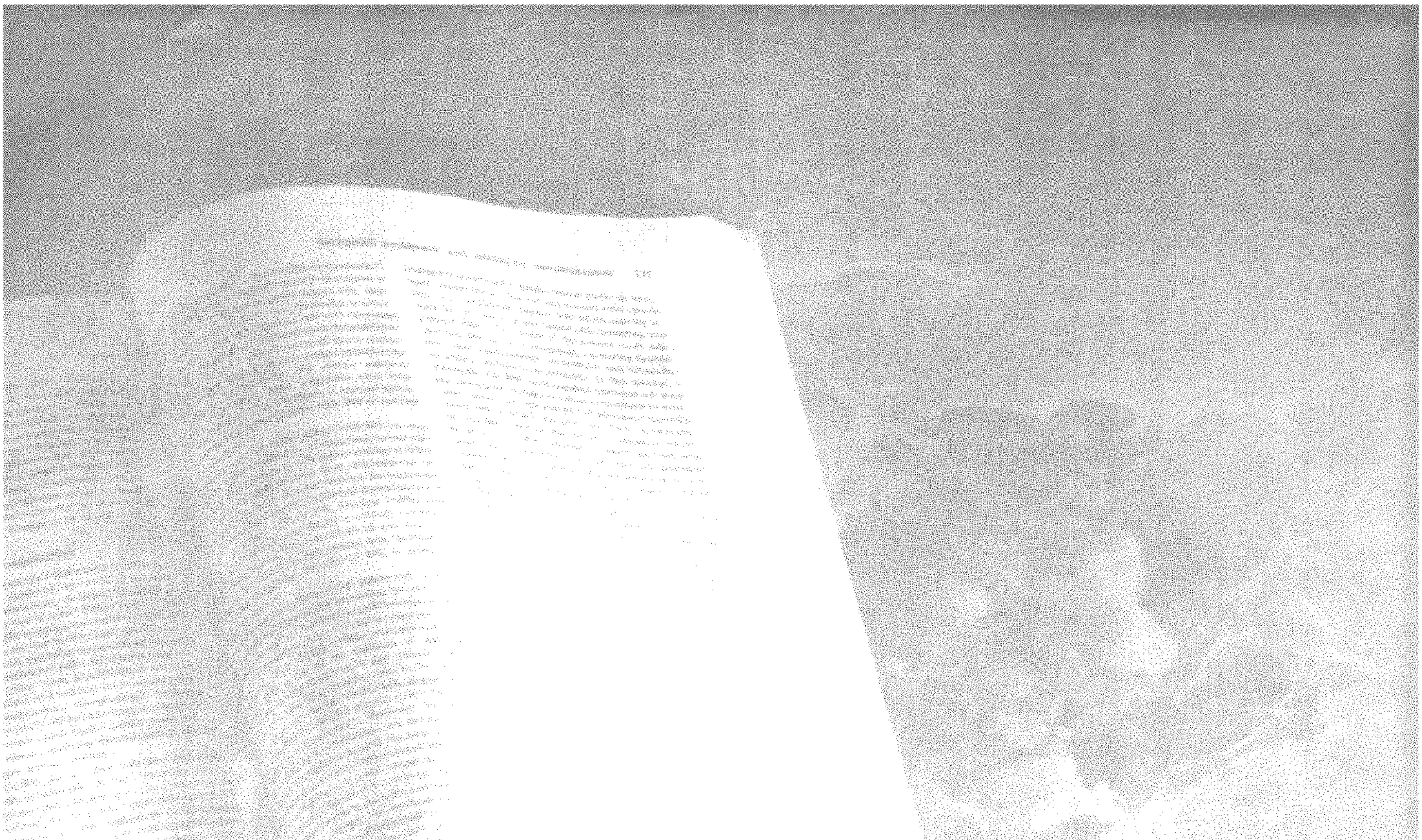

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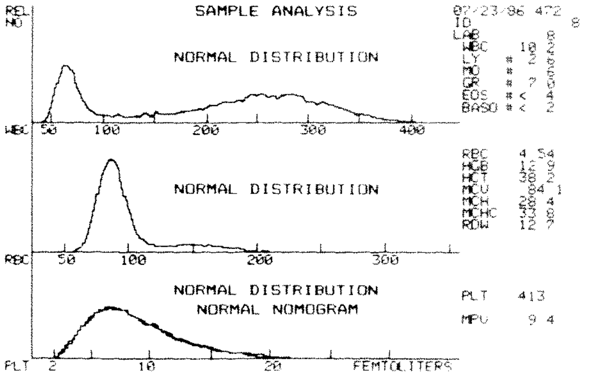

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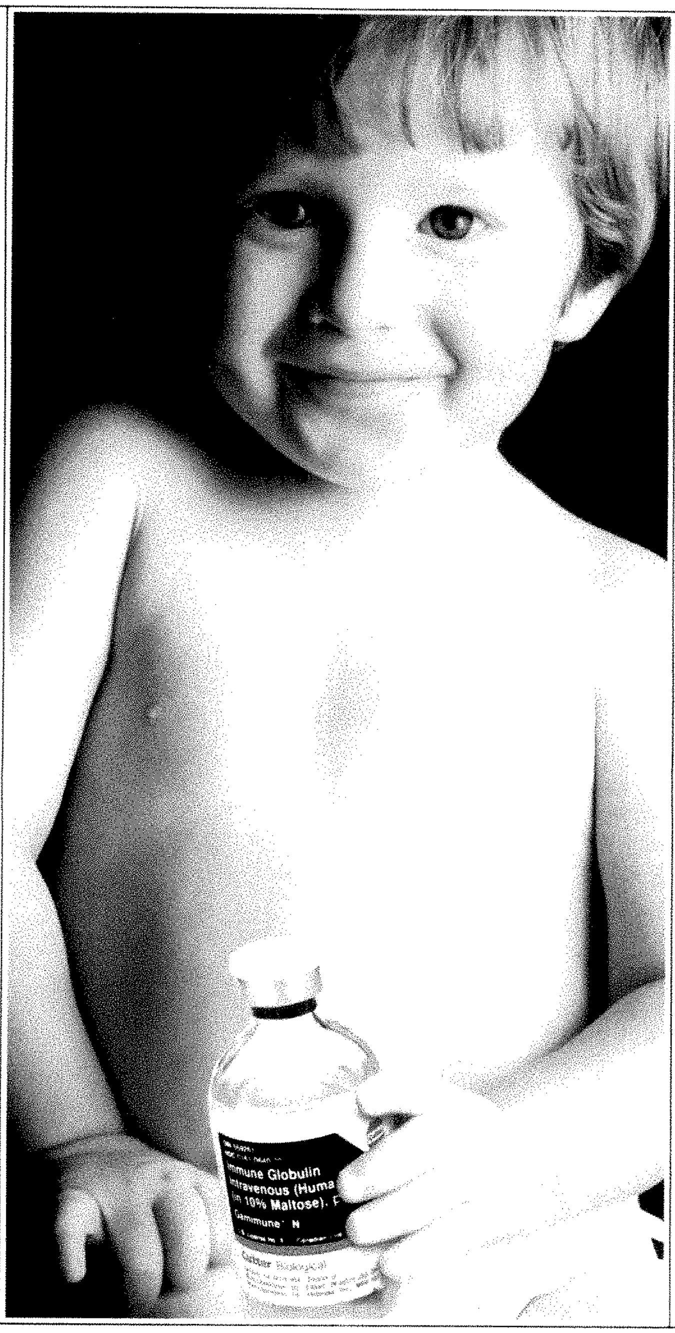

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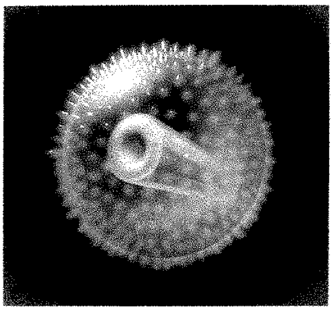

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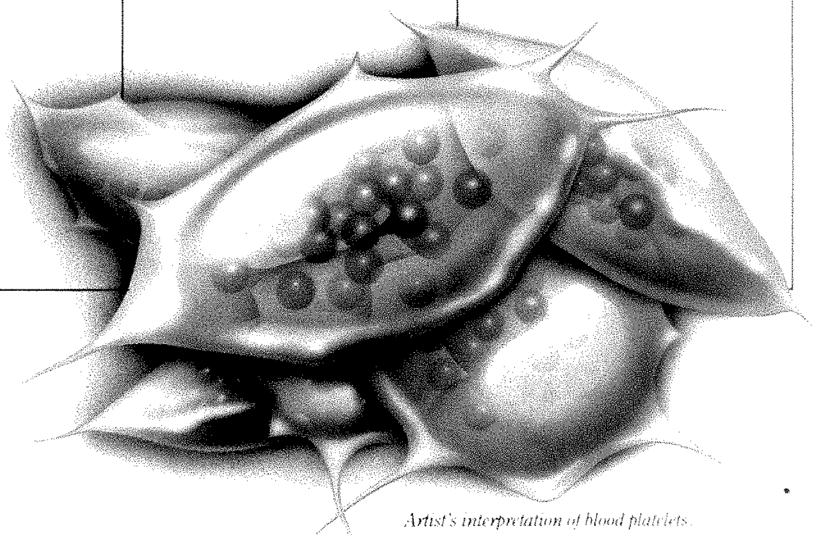

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INDICATIONS AND USAGE

Immunodeficiency Syndromes: *Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.*

Idiopathic Thrombocytopenic Purpura (ITP): *Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.*

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: *Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.*

Drug Interactions: *If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.*

Pregnancy Category C: *Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.*

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: *The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.*

Idiopathic Thrombocytopenic Purpura: *An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.*

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** *increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.*

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. *GAMIMUNE® N Product License Application.* **2.** *Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. Rev Infect Dis 1986;8(Jul–Aug): S382–S390.* **3.** *Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. Transfusion 1986;26(4):394–397.* **4.** *Wells MA, Wittek AE, Epstein JS, et al: Inactivation and partition of human T-cell lymphotrophic virus, type III, during ethanol fractionation of plasma. Transfusion 1986;26(2):210–213.*

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Edited by **B. Hoogstraten, MD, FACP,** Cincinnati, OH

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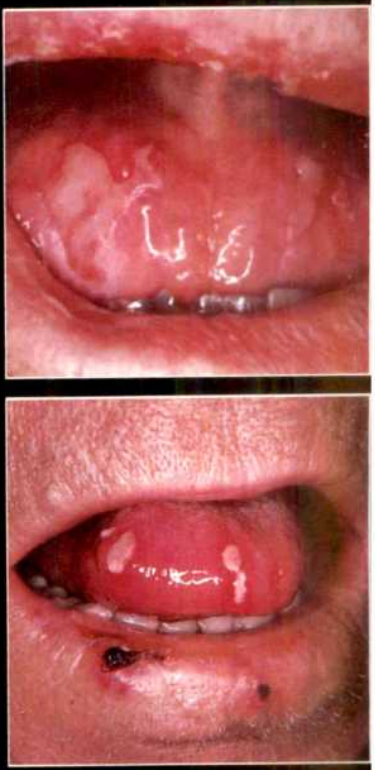

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M² every 8 hours (750 mg/M²/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M²) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated int immunosuppressed syngeneic, weanling mice. Acyclovir was negative in another transformatio system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/k acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic i Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 1 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalia cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses fo mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times th acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. I female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statisticall significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose o 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mous (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtaine did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovi

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should b used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drug are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) steril powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials o Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of th I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache an nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8% experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nause were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebra anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor an clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent advers reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually followin rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathi changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in th intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decreas in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicat that this method may be significantly less efficient in removing acyclovir from the blood. In the event o acute renal failure and anuria, the patient may benefit from hemodialysis until renal function i restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULA OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hour (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years o age, more accurate dosing can be attained by infusing 250 mg/M² at a constant rate over 1 hour, ever 8 hours (750 mg/M²/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above – administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATIO section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M²)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir durin hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentration following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovi The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic wate for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (p approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** Wade, *et al.*, An Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am J. Med. *73*(1A): 229-235, Jul. 20, 198 **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 198 **6.** Z.M. Naib, *et al.*, Cancer Res. *33*:1452-1463, 1973.

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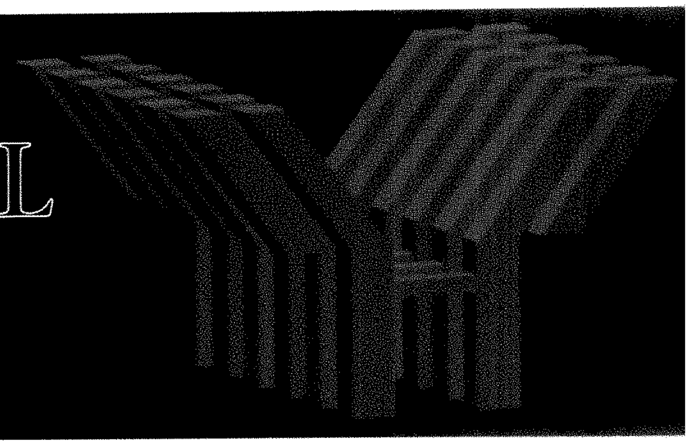

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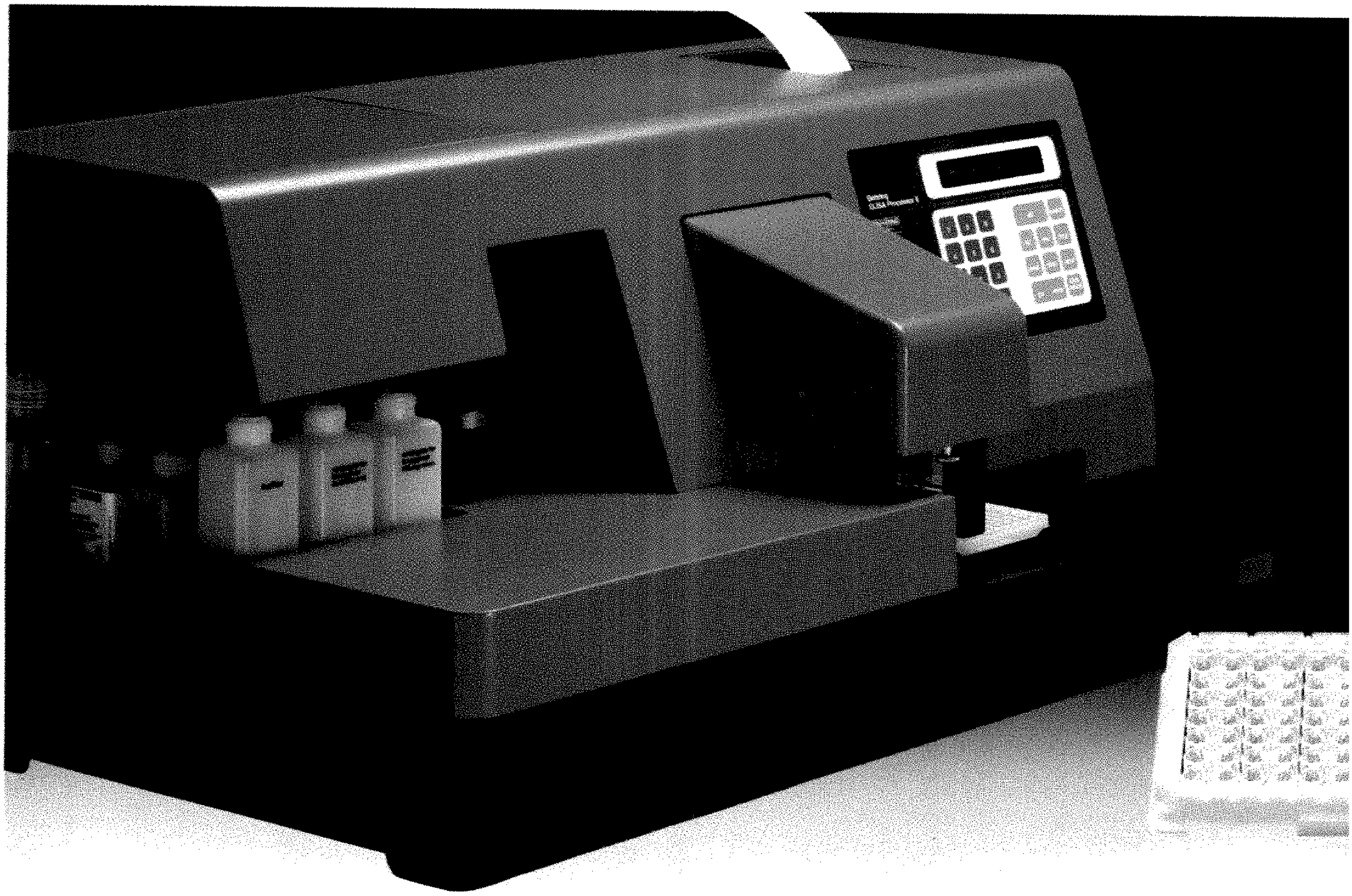

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Today, enzyme immunoassays are expected to possess all of the following important features: reliability, flexibility, accuracy, speed, economy and ease of handling.

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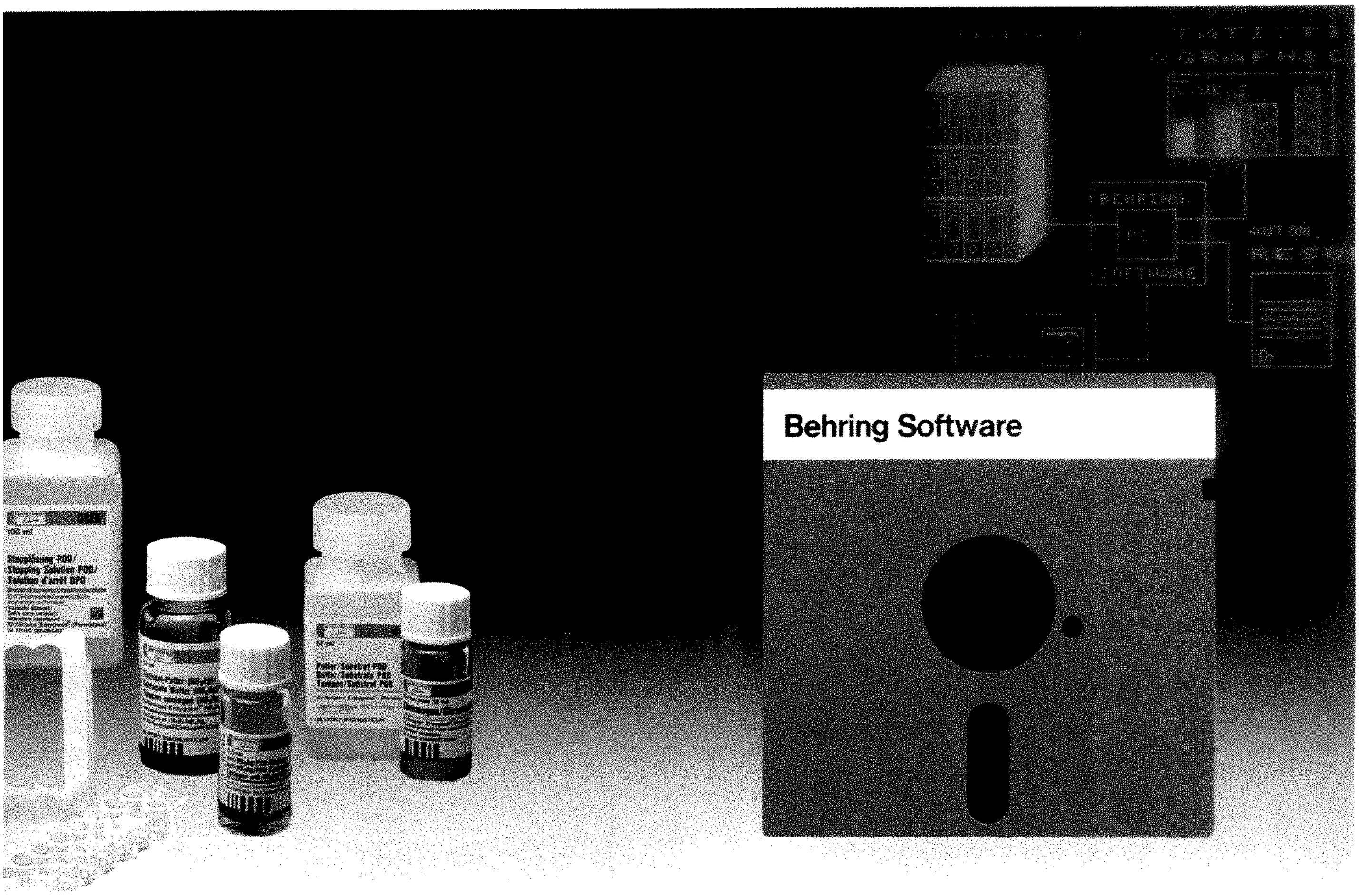

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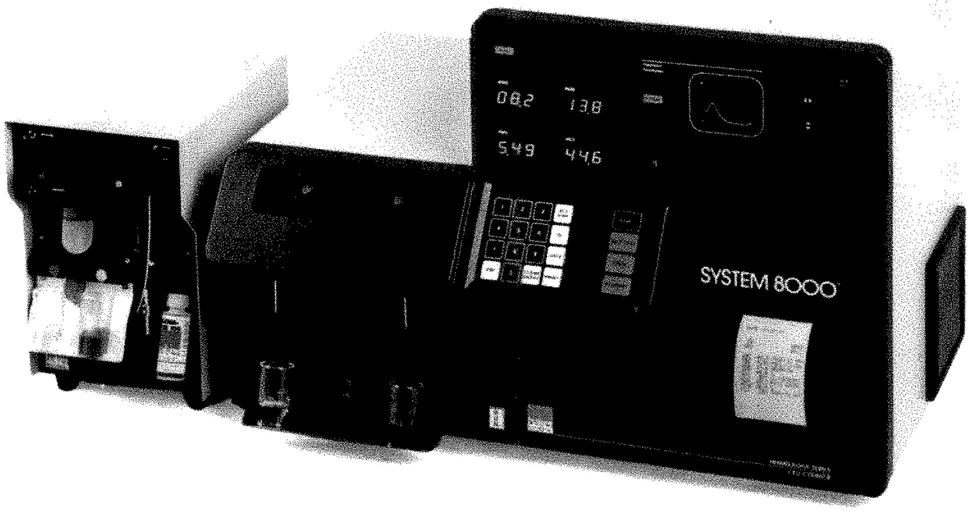

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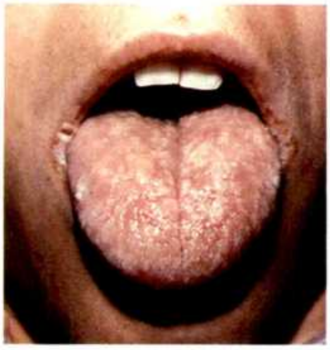
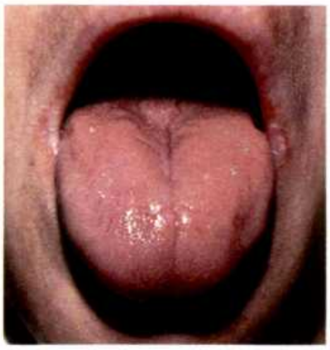

Before treatment.[1]


After treatment with MYCELEX Troche.[1]

High clinical cure[*] rates.

A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

Dramatic improvement in symptoms.

Patients attain a high degree of relief at the end of therapy, as evidenced by the dramatic decrease in severity of glossitis, patches, perleche, pain, and other clinical signs and symptoms of thrush.[4]

Good patient acceptance, minimal side effects.

With MYCELEX Troche, messy suspensions and lengthy swishing are things of the past. MYCELEX Troche has a pleasant, slightly sweet taste. And an entire day's therapy—5 troches—can easily be carried in pocket or purse.

Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole...in the treatment of thrush in any patient population...is both effective and safe."[2†]

[*] Clinical cure is defined as absence of signs and symptoms of the disease.

[†] See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

Mycelex® Troche

(clotrimazole) 10 mg

The only troche for oropharyngeal candidiasis.

MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

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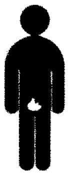
SIX WARNING SIGNS OF KIDNEY RELATED DISEASES

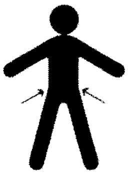


Burning or difficulty during urination

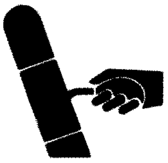
More frequent urination, particularly at night

Passage of bloody-appearing urine

Puffiness around eyes, swelling of hands and feet, especially in children



Pain in small of back just below the ribs (not aggravated by movement)



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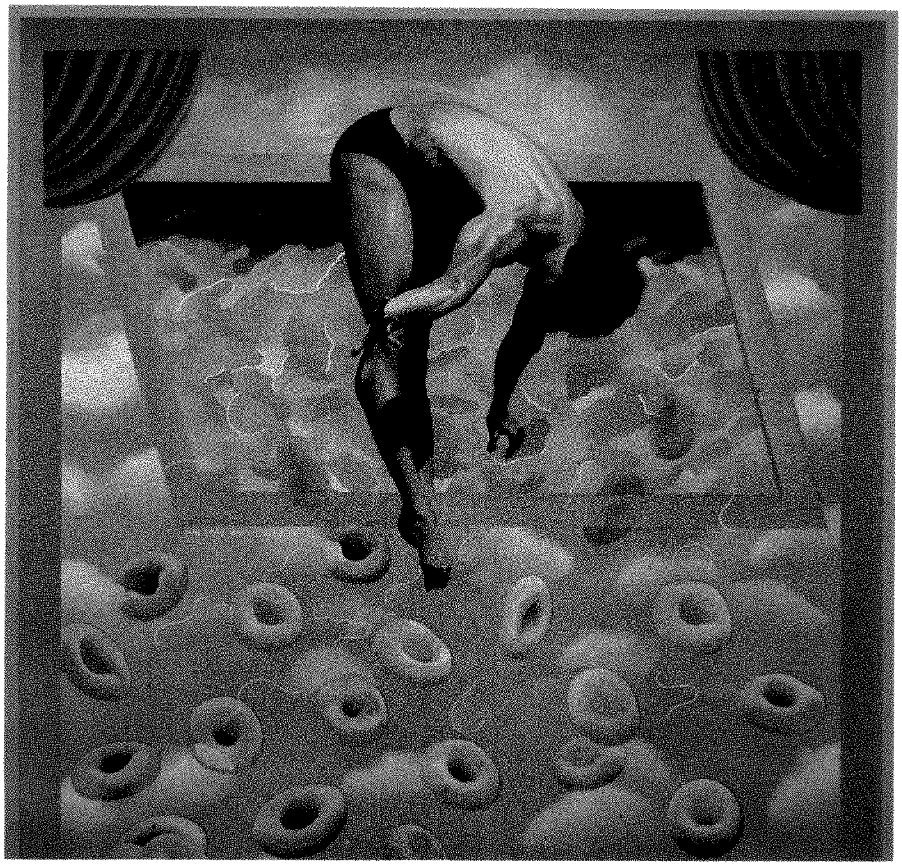

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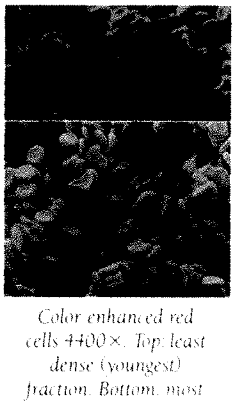

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press:

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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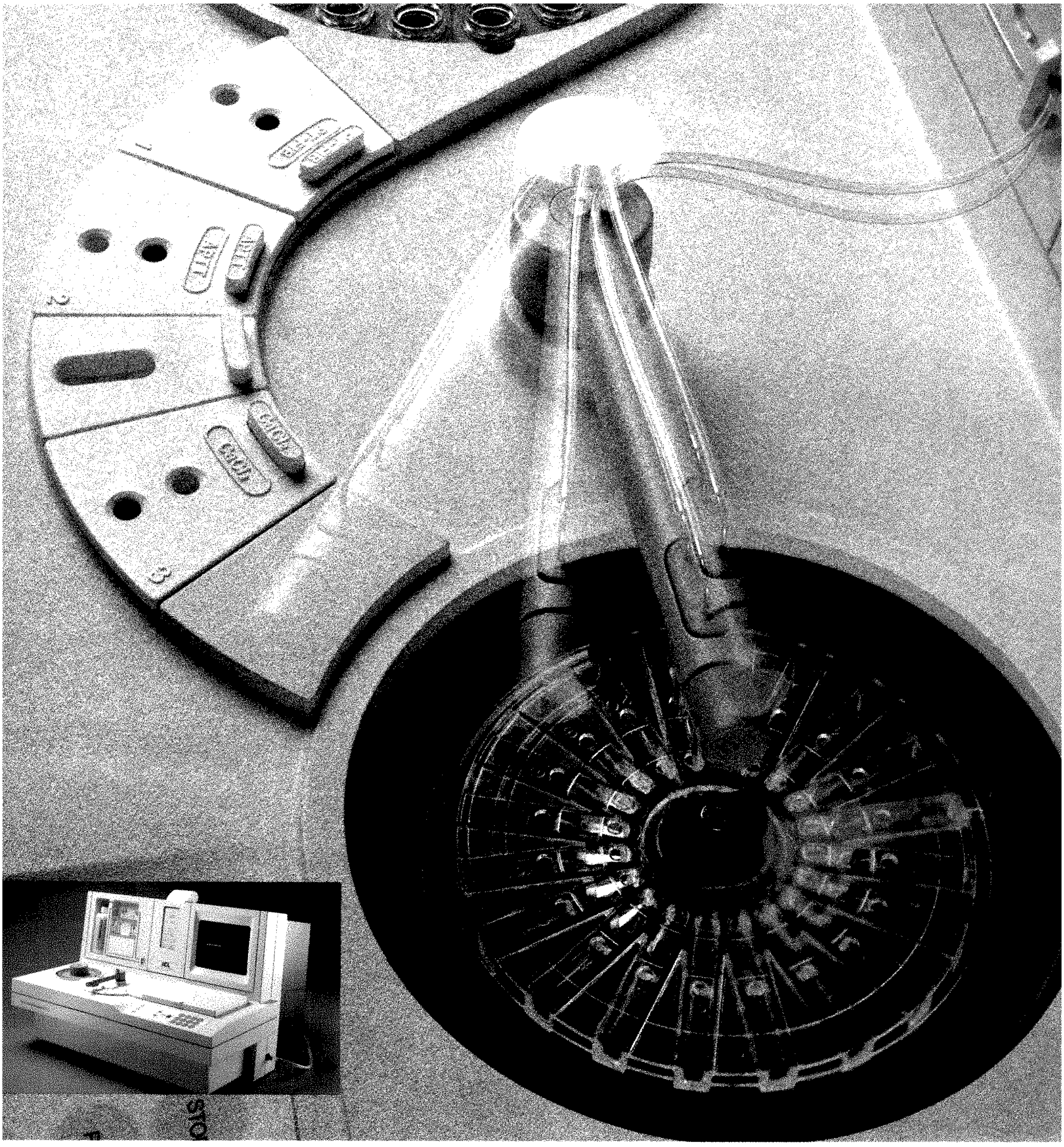

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Cyclophosphamide	350 mg/m²	IV	Weeks 1, 3, 5, 7, 9, 11
Vincristine	1.4 mg/m²	IV	Weeks 2, 4, 6, 8, 10, 12
Bleomycin	10 U/m²	IV	Weeks 4, 8, 12
Prednisone	75 mg	Oral	Daily, dose tapered over the last 15 days
Co-trimoxazole	2 tablets	Oral	Twice daily throughout

† MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; IV = intravenous.

†† Methotrexate given as a 100 mg/m² intravenous bolus, then 300 mg/m² intravenously over 4 hours, followed 24 hours later by folinic acid, 15 mg orally, every 6 hours for 6 doses.

* Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med.* 102:596-602, 1985.

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Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.
(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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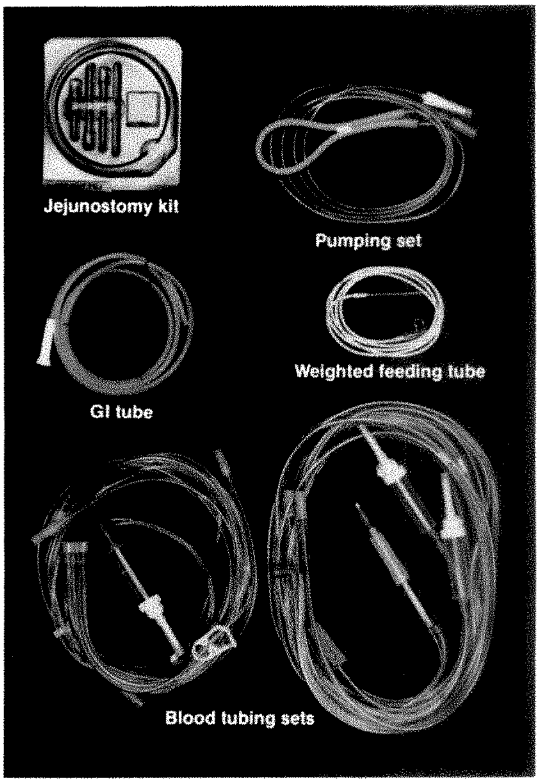

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REVIEW

Clonogenic Cells in Acute Myeloblastic Leukemia

By James D. Griffin and Bob Löwenberg

NORMAL granulocytes, monocytes, and erythrocytes are derived from small numbers of bone marrow progenitor cells that have extensive proliferative capacity.[1-3] In vitro cell culture techniques to identify progenitor cells have been available for 20 years and depend on the ability of these cells to form colonies of fully differentiated cells in semisolid medium in response to specific growth factors.[4-6] These colony assays have been used to investigate in vitro regulation and various biological characteristics of both multipotent and lineage-committed progenitor cells (see reviews 7 to 9). Shortly after the development of normal colony culture techniques these methods were applied to acute myeloblastic leukemias and culture conditions were defined that allowed growth of colonies of leukemic cells.[10-18] In most cases, formation of leukemic colonies was not autonomous, and additition of growth factor(s) from normal leukocytes augmented colony growth. It was surprising, however, that despite the apparent homogeneity of most populations of acute myelogenous leukemia (AML) cells, only a small fraction of cells had the capacity to proliferate in vitro.

These clonogenic leukemic cells have been the subject of considerable investigation, and also the source of some controversy, in the ensuing years. The possibility that these clonogenic cells act as stem cells in vivo to maintain the rest of the leukemic cell population is an intriguing possibility that has potentially important therapeutic significance.[19,20] The identification, culture, growth characteristics, and biological and clinical significance of clonogenic cells in AML will be the subject of this review.

NORMAL HEMATOPOIETIC STEM CELLS

Primitive pluripotent hematopoietic stem cells with the capacity to form all blood elements, including myeloid cells, erythrocytes, megakaryocytes, and lymphocytes, have been demonstrated in mice[21-23] and humans[24,25] using genetic markers. Murine granulocyte/erythrocyte/megakaryocyte multipotent progenitor cells can be readily assayed as spleen colony-forming units (CFU-S), and these cells have been shown to have at least limited self-renewal capacity in secondary colony assay experiments.[1,26] In both mice and humans an in vitro colony assay for a progenitor cell common to granulocytes, erythrocytes, monocytes, and megakaryocytes has been developed (CFU-GEMM),[27-29] and in some circumstances T lymphocytes are also generated.[30] The factors regulating proliferation of this cell are poorly understood, but T cell–derived lymphokines are required in vitro. The CFU-GEMM is a rare cell in normal human bone marrow (approximately 5 to 10 cells per 10^5 mononuclear cells), and despite some similarity to the murine CFU-S, self-renewal capacity is minimal or absent. The progeny of CFU-GEMM cells become committed to one lineage or another, and these committed progenitor cells can be assayed in vitro as erythroid burst-forming units (BFU-E),[31,32] erythroid colony-forming units (CFU-E),[5] granulocyte/monocyte colony-forming units (CFU-GM),[2,4] or megakaryocyte colony-forming units (CFU-Mega).[33] The humoral factors inducing colony formation in each case are partially defined. The CFU-GM cell, for example, proliferates in response to colony-stimulating factors (CSFs), a family of glycoproteins secreted by activated T cells, monocytes, endothelial cells, and certain tumor cells.[34-39] In general, proliferation of any of the committed progenitor cells is associated with differentiation, although colonies of undifferentiated human blast cells have been described.[40] The distinction between various hematopoietic growth factors has not been adequately made in all cases, however, and definition of the range of activity of each factor may require studies with factors produced by molecularly cloned genes. For example, recent studies with recombinant human GM-CSF[41] have shown that this CSF stimulates growth of not only CFU-GM, but also BFU-E and CFU-GEMM.[37,42] It is also likely that direct cell-cell interactions are important in the regulation of hematopoiesis, and stromal cells, endothelial cells, natural killer cells, and T cells may all play roles. However, despite some uncertainty as to the precise regulatory mechanisms of normal hematopoiesis,

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several fundamental principles are clear. True pluripotent stem cells with self-renewal ability are rare cells, and for most progenitor cells proliferation is accompanied by lineage commitment and terminal differentiation.

HEMATOPOIESIS IN AML

Many of these fundamental principles of hematopoiesis are inoperative in AML, a disorder in which there is an accumulation of immature blasts that fail to differentiate to functional granulocytes or monocytes. Normal hematopoiesis is suppressed by either physical displacement of stem cells by leukemic cells or production of inhibitory humoral factors (such as acidic isoferritins[43]), leading to a characteristic pancytopenia of normal blood elements.

Although AML is the result of clonal expansion of a single cell,[44] it is clear that the resulting leukemic cell population is biologically heterogenous despite morphological homogeneity. Studies with ^{3}H-thymidine (^{3}H-TdR) labeling have shown that only a small fraction of AML cells are actively synthesizing DNA in vivo.[45] This small population of proliferating leukemic cells can be identified in vitro by colony assays described below.

TECHNIQUES FOR AML COLONY GROWTH

In the initial attempts to grow leukemic colonies, normal leukocyte feeder cells were added to an agar underlayer as a source of stimulating factors,[6,10] and AML blast cells were plated in a second layer on top of the underlayer. The appearance of truly leukemia derived clones in these cultures has been confirmed by cytogenetic analysis.[46] Other colony-stimulating materials for normal CFU-GM have also been employed for growing AML clonogenic cells, eg, conditioned media from cell lines[47,48] or human placental-conditioned medium (HPCM).[49]

While these methods efficiently induce normal myeloid colony formation, they are often insufficient to permit the maximum outgrowth of AML colonies. In some cases, only single cells surviving in the dish are discerned at the end of culture or no evidence of proliferation is apparent at all.[12,13,15] Frequently small (less than 20 cells) or somewhat larger clusters (less than 40 cells) are produced during culture. Using standard culture techniques, the nongrowing and small cluster-forming groups of patients account for the majority (60% to 70%) of the newly diagnosed cases of AML.[15,50] In fact the poor responsiveness of AML-CFU to stimulation has been explained by their variable and decreased sensitivity to CSF.[49,51]

By analogy with the observations that each type of normal hematopoietic progenitor cell requires a specific colony assay, attempts have been made to develop more efficient AML colony methods. Exposure of the cells to phytohemagglutinin (PHA) during a 15-hour preincubation in suspension and subsequent culturing in a soft agar dish with a leukocyte feeder allowed for the formation of significant numbers of AML colonies of more than 50 cells in the majority of patients with AML [14] In subsequent modifications of the technique, PHA was added directly to one-stage cultures that contained irradiated leukocytes in the agar underlayer and the AML target cells in a liquid overlayer,[16,52] or more recently PHA and HPCM in combination have been used as additions to agar cultures.[53,54] Others have employed PHA-leukocyte-conditioned medium as a stimulus in methylcellulose.[17,55] Repeated feeding of the dishes during the incubation time has been suggested to enhance the colony-forming efficiency as well.[56] These modified cultures are permissive to AML colony formation in 80% to 90% of cases[57,58] and may promote growth of unique clonogenic cells.[49,59] Seven-day colonies are composed of morphologically identifiable blast cells carrying cytogenetic AML markers.[16,52] The blasts of colonies show partial but aberrant maturation when analyzed with combinations of immunological surface markers.[60-63]

T lymphocytes and monocytes are the active components of the leukocyte feeder layer when used in combination with PHA for stimulation of AML-CFU.[64] Natural killer cells may inhibit AML clonogenic growth in vitro.[65] The basis of the higher AML cloning efficiency of the PHA cultures has not been elucidated. The improvement of growth under these conditions may be due to the supply of another inducer that affects AML clonogenic cells, or the release of a cofactor that enhances the colony-forming response to CSFs by causing a shift in the dose response curve.[66] It is less likely that the enhancement of AML colony formation is due to a direct effect of PHA exerted on AML-CFU. PHA as a single ingredient in culture gives poor growth whereas the use of PHA-LCM (which would contain only minimal concentrations of PHA in the final colony cultures) results in effective stimulation.[17,67]

For culture systems with PHA, growth of normal T cells must be avoided by effective T cell separation from the AML marrow/blood sample plated.[16,17,55] T cell marker analysis of colonies is an integral part of the procedure to rule out interfering T cell clone formation. It would be important to identify the growth factors provided by PHA and leukocytes that stimulate the proliferation of AML-CFU: (a) for our understanding of the requirements of AML clonogenic cell proliferation and for delineating abnormalities thereof (eg, for growth factor receptor studies), (b) for separation of these factors from inhibitors, and (c) for standardization of culture techniques for optimal and reproducible application. As PHA-stimulated cells release a wide variety of lymphokines including hemopoietic growth factors that affect different cell types and cell functions, there may be multiple regulators of AML-CFU proliferation including both stimulatory and inhibitory factors. Studies with purified CSFs produced in vitro from cloned genes are likely to provide answers to these questions. Studies with recombinant human GM-CSF (from a gene normally expressed by T lymphocytes) indicate that this factor is a growth promoter of AML cells,[68] but not necessarily the only such factor. Preliminary studies with recombinant human G-CSF and M-CSF indicate that G-CSF is both active in promoting AML-CFU proliferation and further is synergistic with GM-CSF, while M-CSF promotes growth in only occasional cases (Griffin J, unpublished observations) (Table 1).

Table 1. Effects of Recombinant Human CSFs on Growth of AML-CFU

	Growth Promoting Activity For:					
CSF*	CFU-GEMM	BFU-E	CFU-GM	CFU-M	CFU-G	AML-CFU
M-CSF	−	−	−	±	−	−
G-CSF	−	−	+	−	+	+
GM-CSF	+	+	+	+	+	+
IL-3	+	+	+	+	+	+

*Recombinant human CSFs derived from media conditioned by COS cells transfected with full length cDNAs of CSF genes, supplied by Dr Steven Clark, Genetics Institute, Cambridge, MA. CFU-GEMM, multipotent progenitor cell; BFU-E, erythroid burst forming unit, CFU-GM, -M, -G, granulocyte and/or monocyte colony forming unit. CFU-GEMM and BFU-E assay performed in methylcellulose, all others in agar.

BIOLOGICAL CHARACTERISTICS OF CLONOGENIC CELLS IN AML

Cell cycle. As in the case of normal human bone marrow–derived colony-forming cells, it is difficult to determine the in vivo significance of AML-CFU. However, there are several observations that suggest AML-CFU function as leukemic progenitor cells. First, it is likely that AML-CFU are among the group of in vivo cells that are actively proliferating. Thymidine suicide studies show that a very high proportion of AML-CFU are in S phase of the cell cycle and thus are synthesizing DNA in vivo.[19] The thymidine suicide rate is variable, but in some cases is as high or higher than for normal bone marrow CFU-GM. Marie has shown that thymidine labeling index of blasts correlates with the rate of leukemic colony formation.[54] The generation time and duration of S-phase of AML-CFU have been measured by Minden and shown to be similar to normal bone marrow CFU-GM.[69] Thus, AML-CFU are actively cycling and may make up the majority of proliferating cells in vivo.

Self-renewal capacity. The second property of AML-CFU that suggests their role as progenitor cells is self-renewal capacity.[20,70,71] Colonies can be recovered easily from methylcellulose cultures, pooled, and replated to give an estimate of secondary replating efficiency (PE2). Considerable variability in PE2 has been observed among different patients, but PE2 was stable when cells from a single patient were repeatedly tested, even at relapse.[72] PE2 was generally <1% and rarely >10%.[73] Tertiary replating efficiency was always low, and more than 4 generations of colonies have not been observed. The marked variation in PE2 among different patients has been interpreted by McCulloch and colleagues to be due to random determination of self-renewal versus commitment to "differentiation," which as in the case of normal progenitor cells, would be associated with loss of proliferative capacity.[73] When secondary colonies are obtained, they tend to have the same size and morphology as the primary colony.

There is some evidence that more than one colony may be grown in some patients.[74] True colonies often coexist with numerous clusters. Löwenberg has investigated the PE2 of large and small aggregates and found that the PE2 of large colonies is much greater than that for small colonies or clusters.[20] However, secondary colonies were observed occasionally even in the small clusters, although for the most part large secondary colonies were derived only from large primary colonies. The significance of finding large and small colonies is not clear. For normal myeloid progenitor cells, cluster-forming cells are more mature than (and are derived from) colony-forming cells, but neither form secondary colonies. It seems likely that leukemic cluster-forming cells are for the most part derived from colony-forming cells. In some cases the parental cells of colonies and clusters in AML can be distinguished by different surface markers.[20] This may indicate that they represent subsequent differentiation stages, but this remains to be established. Overall, these observations suggest that the leukemic clone may be organized in a fashion similar to normal myelopoiesis, with a hierarchy of progenitor cells generating large numbers of "differentiated" nonproliferative leukemic cells.

DIFFERENTIATION OF CLONOGENIC CELLS IN VITRO

The third property of AML-CFU consistent with their role as progenitor cells is the capacity to undergo differentiation in vitro, at least to a limited extent. It is evident that most daughter cells of AML-CFU exit from the cell cycle and cannot again be induced to proliferate in vitro, a property associated with normal granulocyte/monocyte differentiation. Evidence that AML-CFU may "differentiate" during this process came initially from cytological examination of colony cells[11,47] but cellular morphology was often bizarre and maturation incomplete. More recent data on the abilities of AML-CFU to differentiate in vitro comes from surface marker analysis. A number of investigators have generated monoclonal antibodies that recognize cell surface antigens expressed by normal myeloid cells.[75–80] Some of these antibodies detect antigens that are expressed at discrete stages of normal progenitor cell differentiation and can be used to distinguish cells at various levels of maturation. Many of the differentiation antigens identified by these antibodies are expressed by AML cells, particularly antigens normally expressed by myeloid progenitor cells. Using immunofluorescence and flow cytometry, such monoclonal antibodies have provided the ability to "quantitate" the degree of cellular differentiation of leukemic cells and cell lines in suspension culture.[81–83] Marie studied the acquisition of the granulocyte differentiation antigen MY1 antigen during formation of blast colonies by leukemic cells from eight AML patients.[60] In five of eight cases, the percentage of MY1+ cells in developing colonies increased over a period of two to ten days. In some, but not all, cases this correlated with increased naphthol AS-D chloroacetate esterase (CAE) activity, whereas MY1 expression always correlates well with CAE activity in normal progenitor cells. This was interpreted as showing that many components of granulopoietic differentiation occur in blast colony formation, but different components may be expressed aberrantly. This study was extended by Griffin,[61] who showed that the monocyte antigen MY4 could not be detected on AML-CFU cells prior to in vitro culture, but MY4+ cells could be readily detected in leukemic colonies after several days of culture. Similarly, Wouters and Löwenberg showed that

AML-CFU from patients with AML lacked the granulocyte differentiation antigen B4.3, but acquired this antigen during colony formation.[20] In an additional seven cases, increased numbers of blasts staining for B4.3 or B13.9 antigens were observed after colony formation.[63] In most cases, differentiation was qualitatively and kinetically aberrant when compared with normal CFU-GM cells. For example, in one case expression of an unexpected marker was detected; it was the T lymphocyte differentiation antigen T6. Thus, evidence from several surface marker laboratories has shown that clonogenic cells in AML undergo an aberrant type of "differentiation" during colony formation. In most cases, the resulting cells acquire maturation-associated properties: loss of proliferative potential, loss of self-renewal capacity, and acquisition of new surface proteins that reflect cellular differentiation to a partial extent. This process occurs "spontaneously" in the presence of growth factors but it may be possible to accelerate differentiation by addition of certain differentiation-inducing chemicals such as dimethylsulfoxide, phorbol myristic acid (TPA), or gamma-interferon.[81-86] These compounds are potent inducers of differentiation of leukemic cell lines such as HL-60 and some fresh leukemic cells in suspension culture,[81-86] while GM-CSF is not generally an effective inducer of human leukemic cell lines. More studies are necessary to determine the effects of these agents on clonogenic cells. TPA has been shown to inhibit AML-CFU formation to a greater extent than CFU-GM formation, but this effect may be dose dependent, with very low concentrations of TPA ($<10^{-10}$ mol/L) increasing the secondary plating efficiency.[87]

GROWTH OF AML BLASTS IN SUSPENSION CULTURE

Except in rare cases, growth of AML cells in methylcellulose or agar is self-limited and proceeds for only a short period of time. AML-CFU will also proliferate in liquid suspension culture[88,89] for periods of up to 70 days when stimulated by PHA-LCM, and can be maintained in exponential growth for periods of several weeks. The addition of methylcellulose to liquid cultures inhibited growth.[88] High cell density was required for maximum growth, but this requirement could be replaced by adding blast cell membranes (5×10^6 cell equivalents/mL).[89] The membrane-bound factor has not yet been further characterized.

AML cells have also been grown in long-term marrow cultures, both with and without preformed normal adherent layers. In some cases, but not all, the long-term culture conditions appeared to favor growth of normal progenitor cells rather than AML-CFU.[90] In many cases, adherent layers failed to develop from AML marrows.[91] When AML cells were added to preformed adherent layers, AML-CFU proliferated very briefly and the leukemic cells tended to differentiate rapidly.

SURFACE PHENOTYPE OF CLONOGENIC CELLS IN AML

Recently, considerable progress has been made in the identification of normal progenitor cell surface antigens, and different stages of normal progenitor cells can now be readily distinguished by their phenotypes. These progenitor cell antibodies offer the possibility of identifying a normal counterpart cell for AML-CFU. Three studies have been reported in which AML-CFU have been analyzed with panels of monoclonal antiprogenitor cell antibodies (Fig 1) by complement lysis.[62,92,93] Lange et al studied ten cases with S-13, S8.6, HLA-DR, S16-144, S4.7, and R1B19[62]; Sabbath et al studied 20 cases with HLA-DR, MY9, PM-81, AML-2-23, Mol, Mo2, and MY4[92]; and Löwenberg studied seven cases for expression of S3-13, S4-7, S8.6, S16-144, S16-109, S17-25, B4.3, and HLA-DR antigens.[93] The results of these three studies are summarized in Table 2. Of the 37 cases tested, AML-CFU from all cases expressed one or more of the antigens tested. In most cases, antigens were expressed either by >90% of AML-CFU or by <10% of AML-CFU. However, some antigens, particularly those associated with more mature cells, were expressed heterogeneously by AML-CFU from an individual patient, being found on some, but not all, clonogenic cells. This finding suggests that multiple populations of AML-CFU may exist in some cases. However, low antigen density may account for some of this variability. Unfortunately, no CFU-GEMM–specific antigens have been identified, and all of the CFU-GEMM reactive antibodies employed in these studies also react with more mature progenitor cells. Nevertheless, several antibodies are available, such as PM-81, AML-2-23, and S4-7, which react with intermediate-level progenitor cells, but not with CFU-GEMM. Therefore, reactivity of one of these antibodies with AML-CFU identifies these cells as being the leukemic counterpart of a normal cell at least as mature as the earliest normal progenitor cell expressing that antigen. Thus, 65% and 69% of AML cases had a dominant AML-CFU population that expressed the day 14 CFU-GM antigens PM-81 and S4.7, respectively Thirty per cent of cases had AML-CFU that expressed the day 7 CFU-GM antigen AML-2-23. Interestingly, 8 of 10 cases studied by Lange expressed the late CFU-GM antigen R1B19. Antigens expressed by normal cells only at the blast stage or later (Mol, B4.3, MY3) were not detected on AML-CFU (Table 2). These results suggest that AML-CFU phenotypes

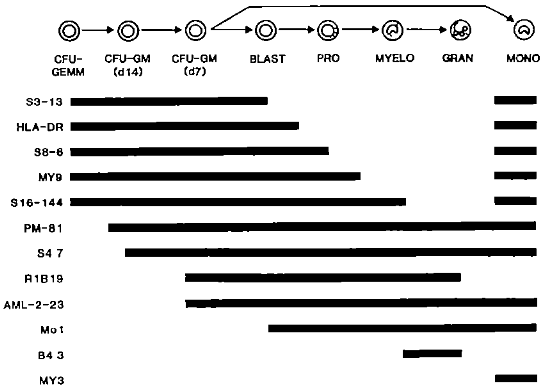

Fig 1. Schematic representation of the distribution of hematopoietic cell surface antigens on normal bone marrow cells. CFU-GEMM, multipotent colony-forming unit; CFU-GM, granulocyte/monocyte colony-forming unit; pro, promyelocyte; gran, granulocyte; mono, monocyte.

Table 2. Surface Antigens of Leukemic Clonogenic Cells

Antibody or Antigen	Least Mature Normal Precursor Cell* Expressing Antigen	Number of AML Cases With >50% Antigen Positive L-CFC	Reference
S3-13	CFU-GEMM	17/17 (100%)	62, 79, 93
HLA-DR	CFU-GEMM	33/37 (89)	62, 92, 93
S8-6	CFU-GEMM	11/16 (69)	62, 79, 93
MY9	CFU-GEMM	19/20 (95)	76, 92
S16-144	CFU-GEMM	16/16 (100)	62, 79, 93
PM-81	day 14 CFU-GM	13/20 (65)	78, 92
S4.7	day 14 CFU-GM	11/16 (69)	62, 79, 93
R1B19	day 7 CFU-GM	8/10 (80)	62, 79
AML-2-23	day 7 CFU-GM	6/20 (30)	77, 92
Mo1	blast/Promyelocyte	1/20 (5)	92, 117
B4 3	metamyelocyte	0/7 (0)	93, 118
MY3	monocytes	0/20 (0)	75, 92

*CFU-GEMM, granulocyte/erythrocyte/monocyte/megakaryocyte colony-forming cell; CFU-GM, granulocyte/monocyte colony-forming cell

vary among different patients. Overall, about ⅓ of cases have had AML-CFU phenotypes comparable to CFU-GEMM (or earlier) cells, while the other ⅔ have expressed phenotypes of committed progenitor cells (day 7 or day 14 CFU-GM). This information is summarized schematically in Fig 2.

Abnormal differentiation in AML is reflected in the considerable variability of AML-CFU phenotypes and in the presence of phenotypes not found in normal cells. Not only the presence or absence of certain determinants but also the density at which these markers are expressed on the clonogenic cells have served to characterize abnormal classes of phenotypes,[94,95] and this could be useful for diagnostic purposes in the future.

COMPARISONS OF SURFACE PHENOTYPES OF AML-CFU WITH THOSE OF AML BLASTS

The phenotypes of the clonogenic cells have also been compared to the phenotypes of the majority of circulating leukemic cells. In the 10 cases studied by Lange,[62] very "early" progenitor cell antigens such as S3-13 and S8-6 were universally expressed by AML-CFU, but were generally lost by the rest of the population in each case. Similarly, in the 20 cases studied by Sabbath,[92] early progenitor cell antigens such as HLA-DR and MY9 were expressed on a significantly higher fraction of AML-CFU than of the total leukemic cell population. In addition, several "late"-appearing antigens, Mo1, Mo2, and MY3, were not expressed by AML-CFU, but were commonly expressed on the total population (9 of 20 cases had greater than 50% of leukemic cells expressing one or more of these antigens). In fact, in each of these 20 cases, the total cell population expressed new differentiation antigens lacking from the less mature AML-CFU population. These results demonstrate that differentiation, although incomplete and often markedly aberrant, is likely to be an ongoing event in vivo in most cases of AML.

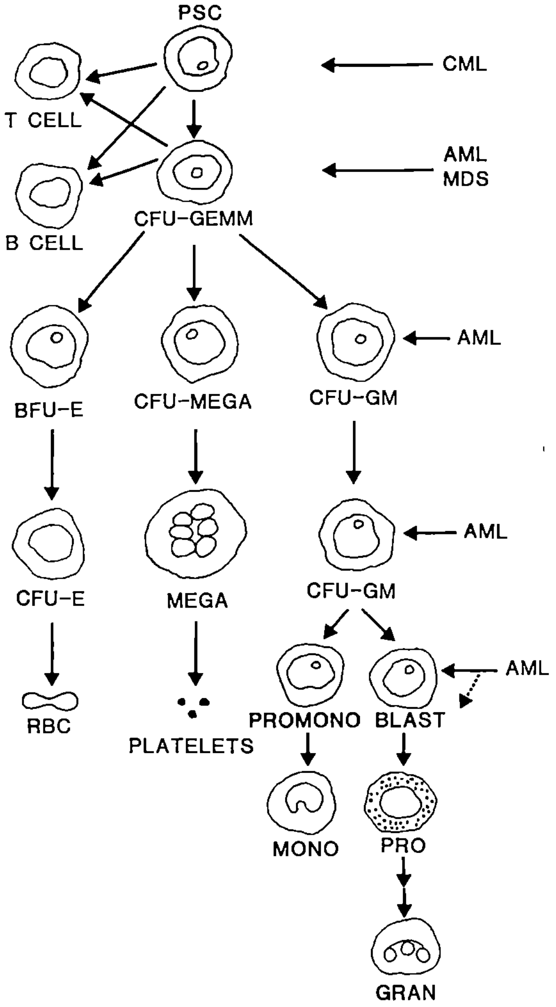

Fig 2. The cell of origin of AML. This is a schematic summary of data from surface marker and G6PD analysis indicating possible sites of origin of AML, chronic myeloid leukemia (CML), and myelodysplastic syndromes (MDS). PSC, pluriopotent stem cell; mega, megakaryocyte (see legend to Fig 1 for other abbreviations). Two CFU-GM cells are shown to indicate early (day 14) and late (day 7) CFU-GM.

The major limitation of this type of analysis is the possibility that the AML-CFU proliferating in vitro is not the most immature leukemic stem cell, but a derivative of a stem cell that does not grow well with available culture systems. Using surface marker analysis to identify different stages of AML-CFU, it should now be possible to optimize culture conditions for growth of the earliest AML-CFU.

THE CELL OF ORIGIN OF AML

Surface marker analysis has shown that AML clonogenic cells may arise at multiple points in hematopoiesis in different patients, and are less differentiated than their progeny in vitro and possibly in vivo. If AML-CFU are representative of the ancestral cell that gave rise to the leukemic clone, then these observations may shed some light on the cell of origin of AML. Unlike chronic myeloid leukemia where genetic[96,97] and glucose-6-phosphate dehydrogenase (G6PD) isoenzyme studies[24] have demonstrated that the cell of origin is a pluripotent stem cell in all cases, similar studies in AML have suggested that some cases arise at the level of pluripotent stem cells while others arise at much later stages.[44] Involvement of the erythroid lineage as well as the granulocyte lineages may be particularly common in cases with a preceding myelodysplastic syndrome. One of the earliest demonstrations of involvement of the erythroid lineage in AML was by Blackstock who described a case in which a chromosomal marker was detected in bone marrow cells that were identified as erythroid by their incorporation of radioactive iron.[98] Even more compelling evidence comes from glucose-6-phosphate dehydrogenase (G6PD) studies. In 1981, Fialkow reported four cases of AML in which two young G6PD heterozygous patients, ages 10 and 11, expressed only a single isoenzyme in leukemic cells, but both isoenzymes in erythrocytes at diagnosis and remission.[44] In contrast, two older patients, ages 69 and 70, had only a single isoenzyme in leukemic cells, erythrocytes, and platelets at diagnosis. In one patient who went into remission, both isoenzymes were later found in erythrocytes, granulocytes, and platelets. In another adult case, analysis of B lymphoid cell lines suggested involvement of the lymphocyte stem cell as well.[99] It has been postulated that the shorter survival of adults compared to children with AML may be due to the more frequent pan-myeloid nature of the illness in adults. G6PD heterozygotes with AML are rare, however, and only a small number of cases have been studied.

One problem of G6PD analysis is that it may underestimate the frequency of involvement of multipotent stem cells. If the leukemic stem cells are limited in either the extent to which they may undergo erythroid differentiation, or in their ability to complete erythroid differentiation, then these cases would be difficult to detect. Considering the profound abnormalities of myeloid differentiation in AML, abnormal erythroid differentiation would not be unexpected. If the leukemic clone supplies 10% of erythrocytes, the ratio of isoenzymes would theoretically be 1.22:1, while if 30% are supplied, the ratio would be 1.86:1. Thus, the contribution of the leukemic clone to the erythron must be substantial for detection by isoenzyme analysis, as ratios as high as 1.5:1 or higher can be seen in normal tissues.[44]

The finding from surface marker analysis that ⅓ to ⅔ of AML-CFU have phenotypes of cells committed to granulocyte/monocyte differentiation[62,92,93] is in agreement with the results of isoenzyme studies that many cases of AML arise at the level of the CFU-GM.[44] The correlation between the two methods is not known, however, and it would be valuable to phenotype AML-CFU from patients heterozygous for G6PD.

PROGNOSTIC VALUE OF AML-CFU

Several investigators have attempted to correlate different patterns of leukemic colony growth with prognosis. As noted previously, the use of a leukocyte feeder layer often generates only scant numbers of colonies, and thus the recovery to normal colony numbers may be used as an indicator of ensuing hematological remission while persistent leukemic growth indicates induction failure.[13,100] Remission colonies exhibit the nonleukemic karyotype.[47] Not only the incidence and karyotype of clone forming cells, but also their buoyant density, thymidine suicide index (as a measure of active cell cycle status of normal colony-forming units), and morphological maturation during colony growth all revert to normal following attainment of complete remission.[11] Recurrence of abnormal growth pattern following attainment of complete remission usually heralds AML relapse.[100]

Various categories of AML colony and cluster growth have been defined in order to test for prognostic significance: (a) no formation of colonies or clusters in culture, (b) mainly small clusters, (c) mainly large clusters, (d) colonies are formed but are rare and exist in the presence of a normal colony-cluster ratio, or (e) colonies coexist with numerous clusters (high cluster-colony ratio). In one large study[12] the categories a, c, and e were associated with a low rate of complete remission. However, in this study diverse chemotherapy programs (often without an anthracycline) were used for remission induction, resulting in an overall complete remission rate of only 36%. Two later studies revealed a poorer prognosis for patients belonging to the large cluster-forming category as compared to the more favorable non-growing and small cluster-forming patient groups (Table 3).[15,48]

In another study an attempt has been made to relate the apparent diminished sensitivity of AML-CFU to CSF[49,51] to the probability of remission induction success. It was suggested that in patients in whom the clone-forming cells exhibited a higher threshold for CSF, the remission rate was reduced.[101] As yet, little information has been collected on the prognostic value of PHA-containing cultures. One study of 19 patients has indicated that the self-renewal capacities of AML-CFU were predictive of a favorable treatment result.[102] Low secondary plating efficiency, assessed by recloning primary colonies in secondary cultures, correlated positively with successful induction. As the abnormal colony-cluster pattern characteristic of patients with AML is frequently expressed in patients with the myelodysplastic syndromes, these abnormalities do not serve as a convenient parameter for assessing progression of these diseases toward AML.[103–108] The newer PHA supplemented assay has been evaluated in a small group of patients with myelodysplastic syndromes and did not provide prognostic information.[109]

Table 3. Pattern of Colony/Cluster Growth in Vitro in Relation to Remission Induction Rate in AML

	Colony Morphology*					
	No Growth		Small Aggregates		Large Aggregates	
Study (Reference)	No of Pts	% CR†	No. of Pts	% CR	No of Pts	% CR
Moore et al[12]	13	15	51	53	44	23
Spitzer et al[15]	21	76	36	75	15	21
Browman et al[48]	12	83	16	69	24	50

*Small aggregates, clusters of 20 cells or less; large aggregates, clusters of more than 20 cells. For the purpose of intercomparison of the three studies, the subdivision of growth patterns was modified according to identical criteria

†CR, complete remission.

SENSITIVITY OF AML-CFU TO CHEMOTHERAPY IN VITRO

Based on the assumption that the clone-forming cells are primarily responsible for perpetual growth, several investigators have attempted to correlate the in vitro sensitivity of AML-CFU to different cytostatic agents with the clinical efficacy of the drugs in vivo.

In pilot studies with anthracyclines, it has been shown that maximal cell kill of AML-CFU is achieved within approximately ten minutes.[110] More prolonged exposures[110] or the continuous addition of the drug to the colony dishes[111] did not add to the cytoreductive effect. Results are identical when fresh or cryopreserved blasts are used.[110]

Cytosine arabinoside and (hydroxy)daunomycin, common drugs in current remission induction schedules, have been evaluated most frequently. The results of the compiled studies are summarized in Table 4. In spite of the methodological variations, it is of note that the correlations between the sensitivity in vitro of AML-CFU to Ara-C and anthracyclines and the ability of these drugs to induce complete remissions are positive in most studies. It is possible that the combined information from parallel sensitivity testing for single drugs[58,112] or from exposure of the cells to multiple drug mixtures[113] permits the most reliable predictions. However, there is substantial overlap in the range of cell kill of AML-CFU between sensitive and resistant leukemias, and absolute discrimination between responders and nonresponders is not possible. The method has been employed to investigate the relative efficacy of certain new anthracycline analogues as well, ie, (hydroxy)daunomycin, tetrapyranyldoxorubicin, and aclacinomycin.[111] The findings of these studies provided evidence for non–cross resistance among these agents.[111] This system has also been used to evaluate differentiation-inducing agents such as interferon[114] and retinoic acid.[115]

At present in vitro sensitivity testing of AML-CFU has not become generally accepted for several reasons. The assay requires a minimum of seven days of culture, which is a drawback for clinical use. The methods employed in various studies are often quite different, so that direct comparisons between the results cannot be made For example, the susceptibility of AML-CFU to cytosine arabinoside has been determined in some cases by adding the drug to colony cultures (continuous exposure),[58,102] but in other studies the cells have been exposed during a short incubation to the drug followed by measuring AML-CFU survival in a colony test (preincubation).[116] The levels for distinguishing in vitro sensitivity from in vitro resistance of AML-CFU (% cytoreduction) vary greatly among different studies, and within one study different cut-off points have been considered for individual agents.[58] Colony techniques and the way of evaluation (colony size and day of culture) in reported studies commonly are different. The choice of the schedules of remission induction chemotherapy and the selection of patients (eg, untreated versus relapsed AML) have been diverse. If one also considers the small numbers of patients included in most of these investigations, that factors other than chemotherapy

Table 4. Reported Studies on Correlations Between in Vitro Sensitivity of AML-CFU to Chemotherapeutic Drugs and in Vivo Response

Chemotherapy* Drug	Ref 116†	Ref 113	Ref 58	Ref 48	Ref 111
Ara-C					
Incubation dose	0.3 ug/mL	—	10^{-5} mol/L	—	—
Incubation time	1 hr	—	continuous§	—	—
% Loss of AML-CFU	>30% <30%	—	>50% <30%	—	—
Patients (n)	6 9	—	10 12	—	—
Pts. with CR (n)	5 2	—	9 3	—	—
	($P = 0.04$)‡		($P < 0.003$)		
Anthracycline					
Incubation dose	0.1 ug/mL DNR	—	10^{-6} mol/L	—	1 ug/mL HDNR
Incubation time	1 hr	—	10 min	—	1 hr
% Loss of AML-CFU	>30% <30%	—	>90% <90%	—	>90% <90%
Patients (n)	9 5	—	13 8	—	10 13
Pts with CR (n)	7 1	—	8 3	—	7 0
	($P = 0.09$)		($P = 0.39$)		($P < 0.001$)
Combined drugs	Ara-C + DNR	Anthracycline+ VCR + Ara-C +Predn	—	Ara-C+ Anthracycline	—
Incubation time	1 hr	1 hr	—	1 hr	—
% loss of AML-CFU	>20% <20%	SI high SI low‖	—	>30% <30%	—
Patients (n)	9 5	5 9	—	15 23	—
Pts. with CR (n)	8 1	4 1	—	11 7	—
	($P = 0.02$)	($P = 0.02$)		($P < 0.02$)	

*Ara-C, cytosine arabinoside, DNR, daunorubicin; HDNR, hydroxydaunorubicin, VCR, vincristin; Predn, prednisone; CR, complete remission.

†Two remission induction failures as a result of hypoplastic death are not included

‡Statistical significance is only indicated for studies involving 15 patients or more; NS, not significant. *P* values determined by Fister exact test.

§Continuous, the drug was added to the colony assays during the entire culture period

‖SI or sensitivity index refers to the relative sensitivity of AML-CFU and indicates the ratio of % survival of CFU-GM *v* % survival of AML-CFU following drug exposure. High SI, more than 1.0; Low SI, less than 1.0.

sensitivity of AML-CFU determine whether a patient will survive remission induction treatment (eg, age), and that all these studies were retrospective, it is clear that the value of testing the chemotherapy sensitivity of AML-CFU is far from definitely established.

CONCLUSIONS

Clonogenic cells in AML have offered a unique opportunity to investigate the concept of stem cells in hematopoietic malignancies. AML-CFU have properties of true stem cells, including high rate of DNA synthesis in vivo, high proliferative capacity in vitro, self-renewal capacity, and ability to differentiate to nonproliferative cells. Surface antigen studies show that AML-CFU are heterogenous among different patients, and are derived from normal counterpart cells at various stages of myeloid differentiation. It may be important to determine the growth factors required for AML-CFU proliferation to optimize in vitro culture, to determine if even earlier stem cells exist, and to better understand how these cells have obtained an apparent proliferation advantage over their normal cellular counterparts. Finally, AML-CFU assays may provide prognostic information through the assessment of self-renewal capacity and determining sensitivity to chemotherapeutic agents. Further investigation in this area is warranted.

REFERENCES

1. Till JE, McCulloch EA: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells Radiat Res 14·213, 1961

2. Bradley TR, Metcalf D. The growth of mouse bone marrow cells in vitro. Austr J Exp Biol Med 44:287, 1966

3. Wu AM, Till JE, Siminovitch L, McCulloch EA: A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J Cell Physiol 69:177, 1967

4. Pluznick DH, Sachs L. The cloning of normal "mast" cells in tissue culture. J Cell Physiol 66.319, 1965

5. Stephenson JR, Axelrod AA, McLeod DL, Shreeve MM: Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc Natl Acad Sci USA 68:1542, 1971

6 Pike BL, Robinson WA Human bone marrow colony growth in agar gel J Cell Physiol 76:77, 1970

7. Quesenberry P, Levitt L: Hematopoietic stem cells N Engl J Med 301:755, 1979

8 Till JE, McCulloch EA: Hematopoietic stem cell differentiation Biochem Biophys Acta 605·431, 1980

9. Ogawa M, Porter PN, Nakahata T: Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review) Blood 61.823, 1983

10 Robinson WA, Kurnick JE, Pike BL: Colony growth of human leukemic peripheral blood cells in vitro. Blood 38:500, 1971

11. Moore MAS, Williams N, Metcalf D. In vitro colony formation by normal and leukemic human hematopoietic cells Characterization of the colony-forming cells. J Natl Cancer Inst 50.603, 1973

12. Moore MAS, Spitzer G, Williams N, Metcalf D, Buckley J· Agar culture studies in 127 cases of untreated acute leukemia: The prognostic value of reclassification of leukemia according to in vitro growth characteristics. Blood 44.1, 1974

13. Bull JM, Duttera MJ, Stashick ED, Nortup J, Henderson E, Carbone PP: Serial in vitro marrow culture in acute myelocytic leukemia. Blood 42:679, 1973

14. Dicke KA, Spitzer G, Ahearn MJ: Colony formation in vitro by leukaemic cells in acute myelogenous leukaemia with phytohaemagglutinin as stimulating factor. Nature 259:129, 1976

15. Spitzer G, Dicke KA, Gehan EA, Smith T, McCredie KB, Barlogie B, Freireich EJ: A simplified in vitro classification for prognosis in adult acute leukemia: The application of in vitro results in remission-predictive models. Blood 48:795, 1976

16. Löwenberg B, Hagemeijer A: Colony formation of human acute myeloid leukemia cells in vitro, in Bentvelzen P, Hilgers J, Yon DS (eds)· Advances in Comparative Leukemia Research. Amsterdam, Elsevier/North Holland, 1977, p 274

17. Buick RN, Till JE, McCulloch EA: Colony assay for proliferative blast cells circulating in myeloblastic leukaemia. Lancet 1·862, 1977

18. Metcalf D: Hematopoietic Colonies. In Vitro Cloning of Normal and Leukemic Cells. Berlin-Heidelberg, Springer-Verlag, 1977

19. Minden MD, Till JE, McCulloch EA: Proliferative state of blast cell progenitors in acute myeloblastic leukemia (AML) Blood 52:592, 1978

20 Wouters R, Löwenberg B: On the maturation order of AML cells; a distinction on the basis of self-renewal properties and immunologic phenotypes. Blood 63:684, 1984

21. Curry JL, Trentin JJ Hemopoietic spleen colony studies. I. Growth and differentiation. Dev Biol 15:395, 1967

22. Curry JL, Trentin JJ, Cheng U: Hemopoietic spleen colony studies. III. Hemopoietic nature of spleen colonies induced by lymph node or thymus cells, with or without phytohemagglutinin. J Immunol 99.907, 1967

23 Abramson S, Miller RG, Phillips RA: The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J Exp Med 145:1567, 1977

24. Fialkow PJ, Jacobson RJ, Papayannopoulou T: Chronic myelocytic leukemia: Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am J Med 63.125, 1977

25. Martin PJ, Naifield V, Hansen JA, Penfold GK, Jacobsen RJ, Fialkow PJ· Involvement of the B lymphoid system in chronic myelogenous leukemia. Nature 287:49, 1980

26 Siminovitch L, McCulloch EA, Till JE: The distribution of colony-forming cells among spleen colonies. J Cell Physiol 62:327, 1963

27. Johnson GR, Metcalf D· Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin Proc Natl Acad Sci USA 74.3879, 1977

28 Hara H, Ogawa M. Murine hemopoietic colonies in culture containing normoblasts, macrophages, and megakaryocytes. Am J Hematol 4.23, 1978

29. Fauser AA, Messner HA: Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53:1023, 1979

30. Messner HA, Izaguirre CA, Jamal N: Identification of T lymphocytes in human mixed hemopoietic colonies. Blood 58:402, 1981

31. Axelrad AA, McLeod DL, Shreeve MM, Heath DS: Properties of cells that produce erythrocytic colonies in vitro, in Robinson WA (ed): Hemopoiesis in Culture. Washington DC, US Government Printing Office, 1974, p 226

32. Gregory CJ. Erythropoietin sensitivity as a differentiation marker in the hemopoietic system: Studies of three erythropoietic colony responses in culture. J Cell Physiol 89:289, 1976

33 Metcalf D, MacDonald HR, Odartchenko N, Sordat B. Growth of mouse megakaryocyte colonies in vitro Proc Natl Acad Sci USA 72:1744, 1975

34. Golde DW, Cline MJ· Identification of the colony stimulating cell in human peripheral blood. J Clin Invest 51:2981, 1972

35. Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura H, Ono M: Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor. Nature 319:415, 1986

36 Nicola NA, Metcalf D, Johnson GR, Burgess AW· Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54:614, 1979

37. Souza L, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K: Recombinant human granulocyte colony-stimulating factor· Effects on normal and leukemic myeloid cells. Science 232:61, 1986

38. DiPersio JF, Brennan JK, Lichtman MA, Speiser BL: Human cell lines that elaborate colony-stimulating activity for the marrow cells of man and other species. Blood 51:507, 1978

39. Metcalf D: The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors Blood 67:257, 1986

40. Nakahata T, Ogawa M. Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies Proc Natl Acad Sci USA 79:3843, 1982

41. Wong GG, Witek JS, Temple PA, Wilkins KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985

42. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang EA, Wong GG, Clark SC. Human recombinant granulocyte-macrophage colony stimulating factor· A multi-lineage hematopoietin. Science 230:1171, 1985

43. Broxmeyer HE, Bognacki J, Dorner MH, de Sousa M· The identification of leukemia-associated inhibiting activity (LIA) as acidic isoferritins: A regulatory role for acidic isoferritins in the production of granulocytes and macrophages. J Exp Med 153:1426, 1981

44. Fialkow PJ, Singer JW, Adamson JW, Vaidya K, Dow LW, Ochs J, Moohr JW: Acute nonlymphocytic leukemia: Heterogeneity of stem cell origin. Blood 57:1068, 1981

45. Clarkson B, Fried J, Strife A, Sakai Y, Ota K, Ohkita T. Studies of cellular proliferation in human leukemia III. Behavior of leukemic cells in three adults with acute leukemia given continuous infusions of ^{3}H-thymidine for 8 or 10 days. Cancer 25:1237, 1970

46. Moore MAS, Metcalf D: Cytogenetic analysis of human acute and chronic myeloid leukemic cells cloned in agar culture. Int J Cancer 11:143, 1973

47. Hoang T, McCulloch EA. Production of leukemic blast growth factor by a human bladder carcinoma cell line. Blood 66:748, 1985

48. Browman G, Goldberg J, Gottlieb AJ, Preisler HD, Azarnia N, Priore RL, Brennan JK, Vogler WR, Winton EF, Miller KB, Grunwald H: The clonogenic assay as a reproducible in vitro system to study predictive parameters of treatment outcome in acute non-lymphoblastic leukemia. Am J Hemat 15:227, 1983

49. Francis GE, Berney JJ, Tuma GA, Wing MA, Hoffbrand AV: Divergent sensitivities of leukemic cells to human placental conditioned medium and leukocyte feeder layers Leuk Res 4:531, 1978

50. Moore MAS: Prediction of relapse and remission in AML by marrow culture criteria. Blood Cells 2:109, 1976

51. Francis GE, Berney JJ, Bol S. Stimulation of human hemopoietic cells by colony stimulation factors: Sensitivity of leukemic cells. Brit J Haemat 41 545, 1979

52. Löwenberg B, Swart K, Hagemeijer A· PHA-induced colony formation in acute nonlymphocytic and chronic myeloid leukemia. Leuk Res 4:143, 1980

53. Dicke KA, Tindle SE, Davis FM, Jagannath S, Tucker S, Lilien M, van Leeuwen P, Verma DA, Vellekoop L: Leukemic cell colony formation in soft agar by bone marrow cells and peripheral blood cells from untreated acute leukemia patients. Exp Haematol 11:341, 1983

54. Marie JP, Zittoun R, Thevenin D, Mathieu M, Perrot JY: PHA-induced blast colonies and kinetic parameters in acute myeloid leukemia. Leuk Res 6:63, 1982

55. Minden MD, Buick RN, McCulloch EA· Separation of blast cell and T-lymphocyte progenitors in the blood of patients with acute myeloblastic leukemia. Blood 54:186, 1979

56 Park CH, Savin MA, Hoogstraten B, Amare M, Hathaway P: Improved growth of in vitro colonies in human acute leukemia with the feeding culture method Cancer Res 37.4595, 1977

57. Swart K, Hagemeijer A, Löwenberg B: Acute myeloid leukemia colony growth in vitro Differences of colony forming cells in PHA-supplemented and standard leukocyte feeder cultures. Blood 59:816, 1982

58. Marie JP, Zittoun R, Thevenin D, Mathieu M, Vigne F: In vitro culture of clonogenic leukaemic cells in acute myeloid leukaemia· Growth pattern and drug sensitivity. Brit J Haemat 55:427, 1983

59. Löwenberg B, Hagemeijer A, Abels J: Detection of the blastic crisis cell clone in chronic myeloid leukaemia. Br J Haematol 59:27, 1985

60. Marie JP, Izaguirre CA, Civin CI, Mirro JH, McCulloch EA: Granulopoietic differentiation in AML blasts in culture. Blood 58:670, 1981

61. Griffin JD, Larcom P, Schlossman SF: Use of surface markers to identify a subset of acute myelomonocytic leukemia cells with progenitor cell properties. Blood 62:1300, 1983

62. Lange B, Ferrero D, Pessano S, Polumbo A, Faust J, Meo P, Rovera G: Surface phenotype of clonogenic cells in acute myeloid leukemia defined by monoclonal antibodies. Blood 64:693, 1984

63. Touw I, Löwenberg B: Variable differentiation of human acute myeloid leukaemia during colony formation in vitro: A membrane marker analysis with monoclonal antibodies. Br J Haematol 59:37, 1985

64. Swart K, Löwenberg B: Feeder cell requirements for leukemia cell colony formation in cultures supplemented with phytohemagglutinin. Cancer Res 44:657, 1984

65. Beran M, Hansson M, Kiessling R: Human natural killer cells can inhibit clonogenic growth of fresh leukemic cells. Blood 61:596, 1983

66. Williams N, van den Engh GJ: Separation of subpopulations of in vitro colony forming cells from mouse marrow by equilibrium density centrifugation. J Cell Physiol 86·237, 1975

67. McCulloch EA, Buick RN, Till JE: Cellular differentiation in the myeloblastic leukemias of man, in Saunders GF (ed)· Cell Differentiation and Neoplasia. New York, Raven Press, 1978, p 211

68. Griffin JD, Young D, Herrmann F, Wiper D, Wagner K, Sabbath KD· Effects of recombinant human GM-CSF on proliferation of clonogenic cells in acute myeloblastic leukemia. Blood 67:1448, 1986

69. Minden M, Major P, Griffin J, Wu A, Kufe D: Generation

time of leukemic blast progenitor cells Cell Tiss Kinetics 16:577, 1983

70. Buick RN, Minden MD, McCulloch EA Self renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. Blood 54:95, 1979

71. Chang LJA, Till JE, McCulloch EA: The cellular basis of self renewal in culture by human acute myeloblastic leukemia blast cell progenitors. J Cell Physiol 102:217, 1980

72. McCulloch EA, Buick RN, Curtis JE, Messner HA, Senn JS: The heritable nature of clonal characteristics in acute myeloblastic leukemia Blood 58:105, 1981

73. McCulloch EA, Izaguirre CA, Chang LJA, Smith LJ. Renewal and determination in leukemic blast populations. J Cell Physiol Supple 1:103, 1982

74. Löwenberg B, Hagemeijer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641, 1982

75. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant myeloid cells J Clin Invest 68 932, 1981

76. Griffin JD, Linch D, Sabbath K, Schlossman SF. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 4:521, 1984

77 Ball ED, Graziano RF, Shen L, Fanger MW: Monoclonal antibodies to novel myeloid antigens reveal human neutrophil heterogeneity Proc Natl Acad Sci USA 79:5374, 1982

78. Ball ED, Graziano RF, Fanger MW A unique antigen expressed by myeloid cells and acute leukemia blast cells defined by a monoclonal antibody. J Immunol 130:2937, 1983

79. Pessano S, Palumbo A, Ferrero D, Pagliardi GL, Bottero L, Lai SK, Meo P, Carter C, Hubbell H, Lange B, Rovera G. Subpopulation heterogeneity in human acute myeloid leukemia determined by monoclonal antibodies Blood 64:275, 1984

80 Ferrero D, Broxmeyer HE, Pagliardi GL, Venuta S, Lange B, Pessano S, Rovera G: Two antigenically distinct subpopulations of myeloid progenitor cells (CFU-GM) are present in human peripheral blood and marrow Proc Natl Acad Sci USA 80.4114, 1983

81. Todd RF, Griffin JD, Ritz J, Nadler LM, Abrams T, Schlossman SF: Expression of normal monocyte-macrophage differentiation antigens on HL60 promyelocytes undergoing differentiation induced by leukocyte conditioned medium or phorbol diester Leuk Res 5:491, 1981

82. Perussia B, Leibman D, Ip SH, Rovera G, Trinchieri G Terminal differentiation surface antigens of myelomonocytic cells are expressed in human promyelocytic leukemia cells (HL60) treated with chemical inducers. Blood 58:836, 1981

83. Ferrero D, Pessano S, Pagliardi GL, Rovera G. Induction of differentiation of human myeloid leukemias: Surface changes probed with monoclonal antibodies. Blood 61:171, 1983

84. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC: Normal functional characteristics of cultured human promyelocytic leukemia cells (HL60) after induction of differentiation by dimethylsulfoxide. J Exp Med 149:969, 1979

85. Koeffler HP, Golde DW. Human myeloid leukemia cell lines. A review. Blood 56:344, 1980

86 Pegoraro L, Abraham J, Cooper RA, Levis A, Lange B, Meo P, Rovera G. Differentiation of human leukemias in response to 12-0-tetradecanoylphorbol-13-acetate in vitro. Blood 55.859, 1980

87. Chang LJ-A, McCulloch EA Dose-dependent effects of a tumor promotor on blast cell progenitors in human myeloblastic leukemia. Blood 57:361, 1981

88. Nara N, McCulloch EA: The proliferation in suspension of the progenitors of the blast cells in acute myeloblastic leukemia Blood 65:1484, 1985

89. Nara N, McCulloch EA: Membranes replace irradiated blast cells as growth requirement for leukemic blast progenitors in suspension culture. J Exp Med 162:1435, 1985

90. Coulombel L, Eaves C, Kalousek D, Gupta C, Eaves A: Long-term cultures of cells from patients with acute myelogenous leukemia (AML): Selection in favor of normal phenotypes in some but not all cases. J Clin Invest 75 961, 1985

91 Schölzel CJM, Löwenberg B: Stimulation of proliferation and differentiation of acute myeloid leukemia cells on a bone marrow stroma in culture. Exp Hematol 13:664, 1985

92. Sabbath KD, Ball ED, Larcom P, Davis RB, Griffin JD Heterogeneity of clonogenic cells in acute myeloblastic leukemia J Clin Invest 75.746, 1985

93. Löwenberg B, Bauman JGJ: Further results in understanding the subpopulation structure in AML: Clonogenic cells and their progeny identified by differentiation markers. Blood 66.1225, 1985

94. Delwel HR, Touw IP, Löwenberg B: A fucose binding lectin (UEA) for characterizing acute myeloid leukemia progenitor cells Blood 68:41, 1986

95. Delwel HR, Touw IP, Löwenberg B: Towards detection of minimal disease: Discrimination of AML precursors from normal myeloid precursors using a combination of surface markers, in Hagenbeek A, Löwenberg B (eds) Minimal Residual Disease in Acute Leukemia, 1986. Boston, Martinus Nijhoff, 1986, pp 68–76

96. Nowell PC, Hungerford DA: A minute chromosome in human chronic granulocytic leukemia Science 132:1497, 1960

97. Rowley JD. Ph^1-positive leukemia, including chronic myelogenous leukemia Clin Haematol 9.55, 1980

98. Blackstock AM, Garson CM: Direct evidence for involvement of erythroid cells in acute myeloblastic leukemia. Lancet 2:1178, 1974

99. Ferraris AM, Raskind WH. Bjornson BH, Jacobson RJ, Singer JW, Fialkow PJ. Heterogeneity of B cell involvement in acute nonlymphocytic leukemia Blood 66 342, 1985

100. Spitzer G, Dicke KA, McCredie KB, Barlogie B: The early detection of remission in acute myelogenous leukemia by in vitro cultures. Brit J Haematol 35:411, 1977

101 Francis GE, Tuma GA, Berney JJ, Hoffbrand AV: Sensitivity of acute myeloid leukemia cells to colony stimulating activity: Relation to response to chemotherapy. Brit J Haemat 49:259, 1981

102. McCulloch EA, Curtis JE, Messner HA, Senn JS, Germanson TP: The contribution of blast cell properties to outcome variation in acute myeloblastic leukemia. Blood 59:601, 1982

103. Greenberg PL, Nichols WC, Schrier SL: Granulopoiesis in acute myeloid leukemia and preleukemia. New Engl J Med 284:1225, 1971

104. Senn JS, Pinkerton PH: Defective in vitro colony formation by human bone marrow preceding overt leukemia. Brit J Haemat 23:277, 1972

105. Greenberg PL, Mara B. The preleukemic syndrome. Correlation of in vitro parameters of granulopoiesis with clinical features Am J Med 66:951, 1979

106. Verma DS, Spitzer G, Dicke KA, McCredie KB. In vitro agar culture patterns in preleukemia and their clinical significance. Leuk Res 3 41, 1979

107. Lidbeck J: In vitro colony and cluster growth in haemopoietic dysplasia (the preleukemic syndrome). Scand J Haematol 25:113, 1980

108. Gold EJ, Conjalka M, Pelus LM, Jhanwar SC, Broxmeyer H, Middelton AB, Clarkson BD, Moore MAS: Marrow cytogenetic and cell culture analyses of the myelodysplastic syndromes: Insights to pathophysiology and prognosis J Clin Oncol 1 627, 1983

109 Senn JS, Messner HA, Pinkerton PH, Chang L, Nitsch B, McCulloch EA, the Toronto Area Preleukemia Study Group: Peripheral blood blast cell progenitors in human preleukemia. Blood 59:106, 1982

110. Buick RN, Messner HA, Till JE, McCulloch EA: Cytotoxicity of adriamycin and daunorubicin for normal and leukemia progenitor cells of man. J Nat Cancer Inst 62:249, 1979

111. Georgoulias V, Iskandar YL, Amesland F, Ribaud P, Mathe G, Jasmin C: In vitro sensitivity of myeloblast clonogenic cells to doxorubicin, aclacinomycin A and 4'-0-tetrapyranyl-doxorubicin: Correlations with clinical responses. J Clin Oncol 2:1215, 1984

112. Park CH, Amare M, Savin MA, Goodwin JW, Newcomb MM, Hoogstraten B: Prediction of chemotherapy response in human leukemia using an in vitro chemotherapy sensitivity test on the leukemic colony forming cells. Blood 55:595, 1980

113. Park CH, Amare M, Morrison FS, Maloney TR, Goodwin JW: Chemotherapy sensitivity assessment of leukemic colony-forming cells with in vitro simultaneous exposure to multiple drugs: Clinical correlations in acute nonlymphocytic leukemia. Cancer Treat Rep 66:1257, 1982

114. Taetle R, Buick RN, McCulloch EA: Effect of interferon on colony formation in culture by blast cell progenitors in acute myeloblastic leukemia Blood 56:549, 1980

115. Douer D, Koeffler HP: Retinoic acid: Inhibition of the clonal growth of human myeloid leukemia cells. J Clin Invest 69:277, 1982

116. Preisler HD: Prediction of response to chemotherapy in acute myelocytic leukemia. Blood 56:361, 1980

117 Todd RF, Nadler LM, Schlossman SF: Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126:1435, 1981

118. Van der Reijden, van Rhenen DJ, Lansdorp PM, van 't Veer MB, Langenhuijsen MMAC, Englefreit CP, von dem Borne AEGK: A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443, 1983

Lysis of Human Fibroblast Colony-Forming Cells and Endothelial Cells by Monoclonal Antibody (6–19) and Complement

By Camille N. Abboud, Reggie E. Duerst, Christopher N. Frantz, Daniel H. Ryan, Jane L. Liesveld, and James K. Brennan

The murine IgG2a monoclonal antibody 6–19 binds to a wide variety of nonhematopoietic cells including human marrow-derived stromal cells but does not bind to marrow or peripheral blood cells. We studied the effects of this antibody and rabbit complement on marrow cells. Fibroblast colonies were eliminated from light density marrow cells by a single incubation with monoclonal antibody 6–19 and complement. The growth and composition of granulocytic and erythroid colonies were unaffected. Specific complement mediated cytotoxicity of the antibody was confirmed on passaged human fibroblasts derived from marrow (more than 99.6% of fibroblasts are killed by a single treatment). Similar results were obtained with human umbilical cord endothelial cells. In addition, such treatment abolished the initiation of Dexter culture stroma. Incubation of bone marrow cell suspensions with this antibody and complement will allow the study of stroma-free marrow cells in long-term liquid cultures.

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HEMATOPOIESIS in man takes place in the extravascular spaces between marrow sinuses. In these locations hematopoietic cells are in close contact with several distinct stromal elements that form the hematopoietic microenvironment.[1] In vivo two cell types have been identified, namely, sinus endothelial cells and adventitial reticular cells, and intimate anatomical relationships between these reticular cells and their hematopoietic counterparts is in keeping with their putative nurturing function.[2] Recently, using the Dexter liquid culture system, the importance of the establishment of a complex adherent layer has been emphasized in both mouse and human long-term marrow cultures.[3] Nevertheless, because several cell types can be identified in such monolayers the individual contribution of each cell type (fibroblast, endothelial cell, macrophage, or adipocytes) to the hematopoietic microenvironment has been difficult to quantitate. Here we report that a murine IgG2a monoclonal antibody (MoAb 6–19), reactive with human neuroblastoma,[4,5] binds to human marrow stromal cells, and can be used to deplete fibroblast colony-forming cells (CFU-F) as well as endothelial cells from human marrow cell suspensions after treatment with complement (C′). Since other progenitor cells (CFU-GM, CFU-E, and BFU-E) are not affected by this treatment, this method should allow the in vitro quantitation of the functional role of stromal cells in long-term cultures by providing a source of marrow cells depleted of fibroblasts, endothelial cells, and Dexter culture stroma

MATERIALS AND METHODS

Cell preparation. Marrow cells were obtained from normal volunteers after informed consent. Light density marrow cell suspensions were prepared as previously described [6] To obtain fibroblasts, light density marrow cells were plated at 2 × 10^6 per mL in McCoy's 5A medium (GIBCO, Grand Island, NY) containing 15% fetal calf serum (FCS) (Hyclone, Logan, UT). Medium was replaced twice a week, and subconfluent monolayers of attached cells were passaged with 0.1% trypsin, 0.02% EDTA, in phosphate-buffered saline (PBS). Dexter stromal cells were obtained as previously described[7] and were similarly passaged three times prior to study unless otherwise specified. Umbilical cord endothelial cells were obtained as described by Wagner et al [8] Human foreskin fibroblasts were obtained from Dr Norbert Roberts and the human embryonic lung fibroblast line MRC-5 was purchased from MA Bioproducts, Walkersville, MD. All fibroblast cultures were maintained in McCoy's medium with 15% FCS. Peripheral blood–derived macrophages were prepared by plating adherent light density cells in DMEM with 10% FCS in flasks for a period of 24 days. Long-term human marrow cultures were initiated as described by Slovick et al.[7]

Bioassays Fibroblast colony assay: light density marrow cells were plated at 2 to 5 × 10^5 per mL in McCoy's 5A and 10% FCS into 35 mm plastic dishes and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Colonies of fibroblasts (CFU-F) were counted in quadruplicate dishes at ten days of culture.[9] Hematopoietic progenitor cell assays· marrow progenitor assays were performed as previously described [10] A single lot of dialyzed GCT-conditioned medium was used as a source of colony-stimulating activity. Granulocytic progenitor cell assays (CFU-GM) were plated at a cell density of 5 × 10^4/mL. Erythroid progenitor cells (CFU-E and BFU-E) were assayed by plating light density marrow cells at 1 × 10^5/mL in dishes containing 2 units of erythropoietin (Connaught step III) per mL of 0 8% methylcellulose Colonies were scored at seven and 14 days of culture. Morphologic analysis of granulocytic colonies was done at day 14 of culture.[6]

Monoclonal antibodies The IgG2a MoAb 6–19 was prepared as described.[5] MoAb 6–19 was purified from ascites by affinity chromatography on a staphylococcus protein A–Sepharose 4B column as described by Ey et al.[11] Monoclonal antibody R1B19 (courtesy of Dr Giovanni Rovera) and common leukocyte antibody GAP 8 3 were directly conjugated to phycoerythrin as described by Ryan et al [12] OKM1 was obtained from the ATCC and was also used as a phycoerythrin conjugate Monoclonal antibodies 6–19 and PC5 were directly conjugated to FITC as previously described by Goding [13]

Immunofluorescence and flow cytometry. Adherent cells were detached with phosphate-buffered saline (PBS) containing 0.1% trypsin and 0 02% EDTA, washed, and resuspended at 2 × 10^7/mL in Puck's saline G, pH 7.6, containing 0.1% sodium azide and 1%

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FCS. Purified MoAb or isotype specific control myeloma protein was added at 5 μg/mL and the cells were incubated for 30 minutes at 4 °C. The cells were washed and incubated in the same solution containing affinity-purified fluorescein-conjugated goat antimouse IgG for 30 minutes at 4 °C. In later experiments, the 6–19 MoAb and PC-5 isotype control directly conjugated to FITC were used. Flow cytometric analysis was performed the same day on a Coulter Epics IV (Hialeah, Fla), using an argon laser with 488 nm excitation wavelength at 500 mW power.[4] Low-angle light scatter was used to gate out dead cells and debris.[14]

Antibody-dependent C′ mediated cytotoxicity. Light density marrow cells were resuspended in medium containing 50% Dulbecco's modification of Eagles medium–50% Ham's F12 (DME-F12), with 15% FCS at 5×10^6/mL. Aliquots of cells were incubated for 15 minutes at 4 °C, with or without MoAb 6–19 (40 μ/mL). Sterile, low endotoxin baby rabbit serum (Pel-Freez) was added as the C′ source, at a final concentration of 20 vol% (1/5 dilution), and the cells were incubated for an additional 30 minutes at 37 °C. The cells were then pelleted and resuspended in McCoy's 5A with 10% FCS and plated in progenitor cell assays. Cytotoxicity assays of cultured cells were performed using a sensitive Hoechst dye-trypan blue exclusion technique.[15] Early passage (two to five) marrow fibroblasts were detached as above, washed, and resuspended in DME-F12 medium containing 15% FCS. The fluorescent dye Hoechst 33342 was added to a concentration of 5 μmol/L and the cells were incubated for 60 minutes at room temperature. The cells were pelleted and suspended in DME-F12, with 15% FCS at concentrations of 5×10^4/mL to 3×10^5/mL. Purified MoAb 6–19 was added to a final concentration of 5 to 40 μg/mL and the samples were incubated for 15 minutes at 4 °C. Sterile, low endotoxin baby rabbit C′ was added to 10 to 20% of the final volume and the cells were incubated for 30 minutes at 37 °C. Cells were then pelleted and resuspended in DME-F12, containing 7.5% fetal calf serum and 0.25% trypan blue and were examined with ultraviolet incident light in a fluorescence microscope to count the viable cells.

RESULTS

Reactivity of MoAb 6–19 with mononuclear cells and stromal cells. Previous studies using immunoperoxidase staining techniques of tumor tissue have indicated positive staining of 6–19 MoAb of stromal elements but not myeloid cell lines such as HL-60, U937, and K562 or lymphoid cell lines Nalm-1 and REH.[5] This led us to compare the staining of 6–19 MoAb to its isotype control PC5 on mononuclear light density cells from blood and marrow (Fig 1). No appreciable specific staining of peripheral blood mononuclear cells nor bone marrow cells was detected. These results are in keeping with the lack of reactivity of this antibody with myeloid and erythroid cells. In order to study the specificity of the reactivity of this antibody to stromal cells similar studies with 6–19 MoAb directly conjugated to FITC were carried out as shown in Fig 2. Definite staining with 6–19 antibody above control occurred with human fibroblasts derived from marrow (A), foreskin (B), or human embryonic lung (C). In addition, Dexter stroma grown in the presence of horse serum and cortisone was found to react with this antibody (2D). No reactivity of 6–19 MoAb occurred with cultured peripheral blood macrophages as indicated in 2E. Finally, we investigated 6–19 binding to human endothelial cells as shown in 2F, and they were positive for that antigen.

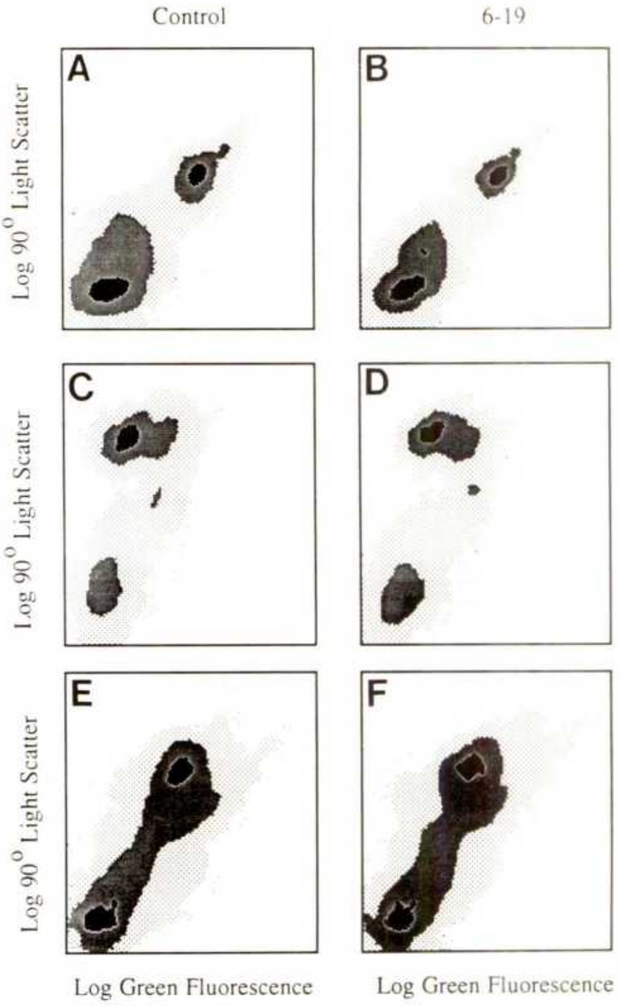

Fig 1. Peripheral blood and bone marrow mononuclear cells stained with 6–19 MoAb. Each histogram displays log green fluorescence *v* log 90 degree light scatter for cells stained with PC5 control antibody (A,C,E) or 6–19 antibody (B,D,F). Ficoll-Paque (Piscataway, NJ) separated peripheral blood mononuclear cells are shown in A and B, dextran-sedimented peripheral blood leukocytes in C and D, and light density bone marrow cells in E and F. Twenty thousand cells were counted in each histogram with the shaded areas of increasing density indicating 2, 16, 128 cells per channel in the 64 × 64 channel two parameter histograms.

Effect of 6–19 MoAb and complement on CFU-F and progenitor cells. In view of the ability of 6–19 MoAb to bind to cultured marrow fibroblasts, we then studied the effects of treatment of marrow light density cells with this antibody and C′ on marrow fibroblast colony growth and granulocytic progenitor cells (Table 1). The treatment with MoAb 6–19 and C′ completely eliminated CFU-F growth from the initial mean of 37 ± 4 CFU-F per 5×10^5 cells, while CFU-GM growth at days 7 and 14 of culture was not affected. In a separate experiment, cytotoxicity studies were carried out in duplicate and fibroblastic, erythroid, and granulocytic colony growth were analyzed. Fibroblast colony growth was abrogated following incubation with MoAb 6–19 and C′ as shown in Fig 3. The effects of MoAb 6–19 and/or

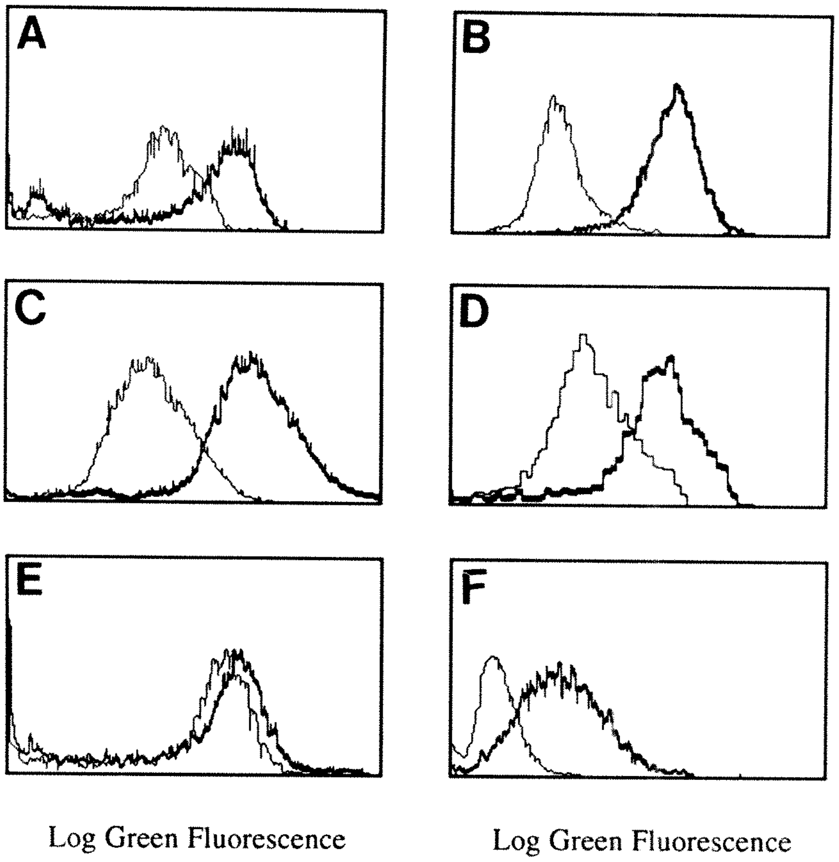

Fig 2. Reactivity of 6–19 MoAb with stromal cell types. Each single parameter histogram displays log green fluorescence *v* cell number for cells stained with PC5 control antibody (light tracing) or 6–19 antibody (heavy tracing). Bone marrow fibroblasts (A), human foreskin fibroblasts (B), MRC-5 cell line (human lung fibroblasts) (C), passaged Dexter stromal cells (D), cultured peripheral blood derived macrophages (E), and human umbilical vein endothelial cells (F) are shown. Ten to 20,000 cells were counted in each histogram. To obtain the histogram in D, cells positive for phycoerythrin-conjugated R1B19 or GAP 8.3 were gated out. The log green fluorescence intensity of the remaining 45% of the cells is displayed (D).

C′ on erythroid and granulocytic colony growth are also shown. In contrast to fibroblast colonies, there was no appreciable decrease in the growth of these progenitors. There was also no detectable alteration in colony subtypes at day 14 between untreated cells and cells exposed to MoAb 6–19 and C′ (neutrophil-monocyte 71% *v* 65%, macrophage 2% *v* 5%, eosinophil 27% *v* 30%).

Cytotoxicity studies on fibroblasts and endothelial cells. Finally, the cytotoxic effect of treatment with MoAb 6–19 and C′ on cultured marrow fibroblasts was determined after staining with Hoechst 33342, as shown in Fig 4. Only a mean of 0.1% of fibroblasts remained viable following incubation with MoAb 6–19 and C′. In a separate experiment human endothelial cells were treated with 5 ug/mL 6–19 MoAb and 10 vol% C′, and survival was determined after staining with Hoechst dye. Survival was 83% in untreated endothelial cells and 82% in cells treated with complement alone, whereas only 11% of cells remained viable after 6–19 and C′ treatment.

Table 1. Effect of Monoclonal Antibody 6-19 and Complement Treatment on Fibroblast Progenitors

Test Conditions		Fibroblast Colonies	Myeloid Progenitors CFU-GM/5 × 10⁴ Cells	
MoAb	C′	CFU-F/5 × 10⁶ Cells	Day 7	Day 14
−	−	37 ± 4	148 ± 30	76 ± 21
+	−	34 ± 3	137 ± 27	78 ± 22
−	+	32 ± 4	127 ± 23	78 ± 20
+	+	0	136 ± 27	79 ± 20

Data represent mean and standard error from seven experiments.

Effect of 6–19 MoAb treatment on Dexter stroma initiation. Long-term human marrow cultures were initiated as previously described[7] using marrow treated with 6–19 MoAb and C′. In two separate experiments, mixed adherent layers failed to develop. Recovery of adherent cells from flasks at 21 days of culture revealed no stromal cells but 89% esterase positive macrophages and 11% chloracetate positive promyelocytes.

DISCUSSION

Several studies have indicated that a special relationship of stromal cells to hematopoietic cells is essential for the orderly growth and differentiation of hematopoietic progenitors.[3,16] Recently, with the use of the Dexter long-term liquid culture system, the study of the contribution of the microenvironment to normal as well as abnormal hematopoiesis has been possible.[3,16,17] While marrow fibroblasts are not the only cells that contribute to the adherent layer composition, they are a major element. Endothelial cells, macrophages, lymphocytes, adipocytes, and ill-defined blanket cells[3] are other accessory cells present in Dexter cultures. The exact relationship of these cells to the anatomically defined adventitial reticular cells lining the marrow sinuses is unclear. The quantitation of the individual contribution of each cell type to the nurturing function of the in vitro microenvironment has been impossible, since separation of stromal cells from

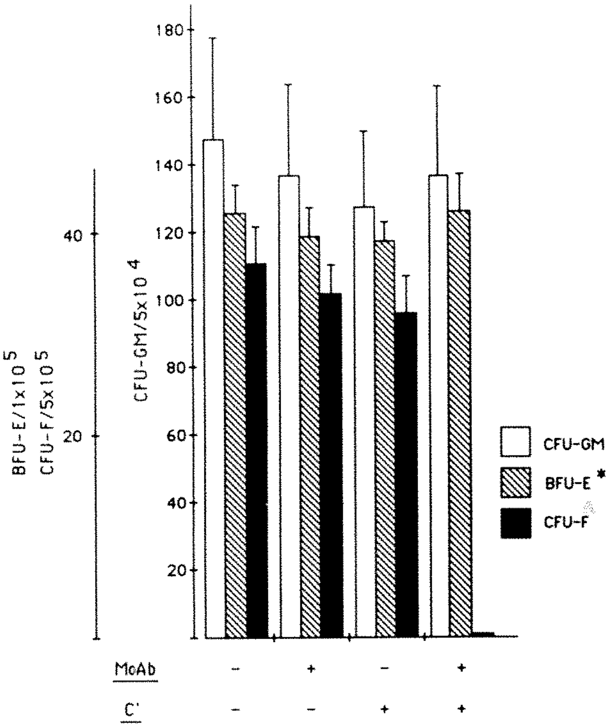

Fig 3. Effect of MoAb 6-19 and C′ treatment on erythroid and granulocytic progenitor growth *v* fibroblast colony growth. Note the complete elimination of CFU-F growth when 6-19 and C′ were used together. Data represent mean and standard error from eight replicate dishes of two duplicate treatments with MoAb and C′. *Results for CFU-E were comparable to those of BFU-E.

marrow by adherence to plastic will usually eliminate monocytes as well as fibroblasts.[9] In the studies described here, we have demonstrated that the MoAb 6-19 and complement can be used to selectively deplete human marrow cells of stromal cells, without affecting the hematopoietic progenitors. Further, we have shown that 6-19 MoAb reacts with stromal cells in Dexter cultures as well as with human umbilical cord endothelial cells. This will allow investigators to prepare human progenitor cells free from all stromal precursors, and hence study the effects of individual stromal cell types in long-term cultures on progenitor survival. A recent report by Charbord et al[18] raises the possibility that hematopoietically active stromal layers in human long-term marrow cultures are different from fibroblasts and endothelial cells based on their reactivity with two MoAb that bind smooth muscle actin. The identity of these cells and their relationship to the adventitial reticular cells is unclear. The demonstration that 99.9% of marrow-derived fibroblasts and 100% of fibroblast colony-forming cells are killed by incubation with MoAb 6-19 and C′ suggests that both cells express the antigen to which MoAb 6-19 binds and that these cells are of similar lineage. Moreover, it appears that light density bone marrow cells that are required to produce hematopoietic stroma express the antigen recognized by 6-19 MoAb, because marrow treated with 6-19 MoAb and C′ was unable to form stroma. The presence of the 6-19 antigen on human endothelial cells in addition to Dexter stroma but not macrophages further underscores the difference between accessory cells such as monocytes and macrophages from stromal cells. The difference in surface structure of stromal cells and hematopoietic progenitor cells is further underscored by a study of Ebell et al,[19] in which depletion of marrow stromal elements (fibroblasts, endothelial cells, and adipocytes) occurred in marrow grafts separated by soybean agglutinin (SBA). Hematopoietic stem cells were SBA negative while the stromal elements and most mature hematopoietic cells were segregated in the SBA positive fraction. The availability of an antibody that effectively removes marrow stromal cells will allow investigators to study the function of these cells and their interaction with other accessory cells in long-term culture.

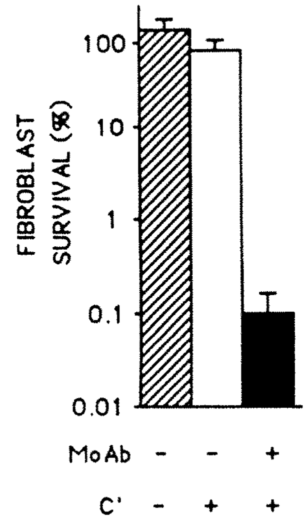

Fig 4. Cytotoxicity of MoAb 6-19 and C′ on human marrow fibroblasts. Data represent mean and standard error from four different experiments.

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REFERENCES

1. Wilson FD: Hematopoietic stroma and microenvironment, in Baum SJ (ed): Current Methodology in Experimental Hematology (Biblthca hematol, No 48). Basel, Karger, 1984, pp 210-292

2. Lichtman MA: The ultrastructure of the hemopoietic environment of the marrow: A review. Exp Hematol 9:391, 1981

3. Wright DG, Greenberger JS (eds): Long-term Bone Marrow Culture (Kroc Foundation Series vol 18). New York, Alan R. Liss, 1984

4. Frantz CN, Duerst RE, Ryan DH, Gelsomino NL, Constine LS, Gregory PK: Anti-neuroblastoma monoclonal antibodies which do not bind to bone marrow cells, in Evans AE (ed): Advances in Neuroblastoma Research. New York, Alan R. Liss, 1985, pp 485-499

5. Frantz CN, Deurst RE, Ryan DH, Constine LS, Gelsomino N, Rust L, Gregory P: A murine IgG2a monoclonal antibody that binds to many nonhematopoietic cell types. Hybridoma 5:297, 1986

6. Slovick FT, Abboud CN, Brennan JK, Lichtman MA: Modu-

lation of in vitro eosinophil progenitors by hydrocortisone: Role of accessory cells and interleukins. Blood 66:1072, 1985

7. Slovick FT, Abboud CN, Brennan JK, Lichtman MA: Survival of granulocytic progenitors in the non-adherent and adherent compartments of human long-term marrow cultures. Exp Hematol 12:327, 1984

8. Wagner DD, Olmsted JB, Marder VJ: Immunolocalization of von Willebrand Protein in Weibel-Palade bodies of human endothelial cells. J Cell Biol 95:355, 1982

9. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MAS: Characterization of human fibroblast colony-forming cells (CFU-F) and their progeny. Blood 56:289, 1980

10. Abboud CN, Brennan JK, Barlow GH, Lichtman MA: Hydrophobic adsorption chromatography of colony-stimulating activity and erythroid-enhancing activity from the human monocyte-like cell line, GCT. Blood 58:1148, 1981

11. Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochem 15:429, 1978

12. Ryan DH, Kossover S, Mitchell S, Frantz C, Hennessy L, Cohen H: Subpopulations of CALLA positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. Blood 68:417, 1986

13. Goding JW: Conjugation of antibodies with fluorochromes: Modifications to the standard methods. J Immunol Meth 13:215, 1976

14. Loken MR, Stall AM: Flow cytometry as an analytical and preparative tool in immunology. J Immunol Meth 50:85, 1982

15. Duerst RE, Frantz CN: A sensitive assay of cytotoxicity applicable to mixed cell populations. J Immunol Meth 82:39, 1985

16. Singer JW, Keating A, Wright TN: The human hemopoietic microenvironment, in Hoffbrand AV (ed): Recent Advances in Hematology (No 4). New York, Churchill Livingstone, 1985, pp 1–24

17. Dexter TM, Moore MAS: In-vitro duplication and cure of hemopoietic defects in genetically anemic mice. Nature 269:412, 1977

18. Charbord P, Gown AM, Keating A, Singer JW: CGA-7 and HHF, two distinct monoclonal antibodies that recognize muscle actin and react with adherent cells in human long-term bone marrow cultures. Blood 66:1138, 1985

19. Ebell W, Castro-Malaspina H, Moore MAS, O'Reilly RJ: Depletion of stromal cell elements in human marrow grafts separated by soybean agglutinin. Blood 65:1105, 1985

Sl/Sl^d Mouse Bone Marrow Stroma In Vitro Contains an Active Radiation-Sensitive Inhibitor of Normal Hemopoiesis

By Kenneth S. Zuckerman, Charles W. Prince, and Maria Ribadeneira

Sl/Sl^d mice have a defective hemopoietic microenvironment. It has been assumed, based upon previous studies, that the primary abnormality in these mice is simply lack of a necessary supportive or inductive material within the hemopoietic stroma. We used in vitro long-term bone marrow cultures to characterize further the nature of the hemopoietic microenvironmental defect in Sl/Sl^d mice. Sl/Sl^d mouse bone marrow cells consistently produced <10% of the total hemopoietic cells and multipotent and unipotent hemopoietic progenitor cells produced in cultures of marrow from normal, congenic +/+ mice. If fresh Sl/Sl^d and +/+ marrow cells were mixed prior to establishing long-term marrow cultures, there was a direct correlation between number of Sl/Sl^d cells added and degree of inhibition of +/+ hemopoiesis. A pre-established, confluent Sl/Sl^d adherent stromal layer inhibited hemopoiesis by fresh +/+ marrow cells by nearly 70%, as compared with dishes with irradiated +/+ or no stroma. This inhibitory effect was abrogated by irradiation of the Sl/Sl^d stroma prior to addition of the fresh +/+ marrow cells. Similarly, unirradiated, but not 9 to 200 Gy irradiated Sl/Sl^d stroma inhibited proliferation of the factor-dependent FDC-P1 hemopoietic progenitor cell line. Thus, the Sl/Sl^d hemopoietic microenvironment actively inhibits hemopoiesis in vitro, and this inhibition can be at least partially eliminated by irradiation of the Sl/Sl^d stroma.

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THE mutant Sl/Sl^d mouse has a hereditary macrocytic anemia and diminished production of cells of other hematologic lineages, which has been demonstrated quite conclusively to result from a defective hemopoietic microenvironment.[1–13] Sl/Sl^d hemopoietic progenitor cells removed from their natural environment and transplanted into irradiated normal mice form macroscopic colonies on the surface of spleens of recipient mice and cure radiation-induced hemopoietic aplasia.[1–11] Sl/Sl^d mouse bone marrow and spleens do not support macroscopic colony formation, and the Sl/Sl^d hemopoietic deficiency is not cured by transplantation of congenic normal (+/+) or other histocompatible normal hemopoietic cells.[1–11] However, hemopoiesis in Sl/Sl^d mice can be restored to normal or nearly normal by subcutaneous or intraperitoneal implantation of intact +/+ mouse spleens or femurs, which subsequently become populated with hemopoietic cells and become a major, if not the main, source of mature hemopoietic cells produced by the mouse.[4,10,11]

It is of considerable interest that the basic Sl/Sl^d hemopoietic defects are reproducible in vitro in long-term bone marrow cultures, although reports differ with respect to the degree of the abnormalities that are observed in vitro.[14–18] Dexter and Moore[15] found that a pre-established Sl/Sl^d stroma did not support hemopoiesis by fresh bone marrow cells from W/W^v mice, which have a known defect of hemopoietic progenitor cells, but a normal microenvironment[3–6]; but a W/W^v stroma supported hemopoiesis by fresh Sl/Sl^d marrow cells. The purpose of our studies was to use long-term bone marrow cultures to better characterize the functional defect of the Sl/Sl^d mouse hemopoietic microenvironment.

MATERIALS AND METHODS

Mice. WCB6F1-(+/+) and WCB6F1-(Sl/Sl^d) mice were obtained from Jackson Laboratories (Bar Harbor, Me) and were maintained by the Department of Comparative Medicine and the Animal Services Department of the University of Alabama at Birmingham. The mice used for these experiments were seven to 16 weeks old.

Long-term bone marrow cell cultures. Long-term bone marrow cell cultures were established and maintained using our previously described modifications[20,21] of the methods of Dexter and his colleagues.[22,23] Bone marrow cells were obtained from the femurs and tibias of the mice, dispersed into a single-cell suspension, and cultured in 60 mm plastic tissue culture dishes (Corning Glass Works, Corning, NY) at a final concentration of 16 × 10^6 nucleated cells in 5 mL of medium, unless otherwise specified. The cultures were maintained in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO, Grand Island, NY), supplemented with 10% horse serum and 10% defined fetal bovine serum (Sterile Systems, Logan, Utah), 10^{-6} mol/L hydrocortisone succinate (Sigma Chemical Co, St. Louis, Mo), 2 mmol/L L-glutamine, penicillin 50 U/mL, and streptomycin 50 ug/mL (GIBCO). The cultures were incubated at 33 °C in an atmosphere of 5% CO_2 in air and were changed weekly by removing all of the medium and nonadherent cells and replacing with fresh supplemented medium alone. The cultures were not recharged with fresh bone marrow cells, with the exception of the experiments in which the ability of an irradiated stromal layer to support growth of fresh +/+ marrow cells added one to 21 days after irradiation was being assessed. Using these techniques we were able to monitor the total cumulative nonadherent hemopoietic cell production, which was calculated simply by adding the number of nonadherent cells recovered on a given day to the sum of all cells recovered during each of the preceding weeks, beginning with the second week. Cells recovered after only 1 week of culture were not included in these totals, since it was impossible to determine how many were simply residual cells from the 16 × 10^6 nucleated cells inoculated into the cultures initially. Primary cultures were maintained for 3 to 4 weeks prior to irradiation in order to permit a confluent or nearly confluent adherent stromal level to be established and to determine whether there was active hemopoiesis demonstrable

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Assays of hemopoietic progenitor cells produced by murine long-term bone marrow cultures Multipotent (CFU-GEMM or CFU-Mix), erythroid (BFU-E), megakaryocytic (CFU-M), and granulocyte-macrophage (CFU-GM) progenitors in the nonadherent cell population removed from the long-term marrow cultures each week were assayed in plasma clot cultures, as described previously.[20,21] Erythropoietin 3 U/mL was used in all CFU-GEMM and BFU-E cultures, and 10% pokeweed mitogen-stimulated spleen cell–conditioned medium was used as a stimulant in all of the progenitor cell assays.

Radiation of long-term bone marrow cultures. All culture plates in an experiment, including those that were to serve as control cultures that received no radiation, were removed from the incubator and transported to the Comprehensive Cancer Center's Small Animal and Tissue Culture Radiation Core Facility. Groups of 3 or 4 culture dishes at a time were irradiated in a Gammacel 1000 unit (Atomic Energy of Canada Limited, Kanata, Ontario), which delivers a dose rate of 5.2 Gy/min. Cultures were irradiated in a single session with 0, 9, 50, or 200 Gy. Then the medium in all of the dishes was replaced with fresh medium, and the cultures were returned to the incubator.

Studies of the effects of stroma from long-term bone marrow cultures on growth of the hemopoietic growth factor–dependent FDC-P1 cell line. The FDC-P1 cell line, which was derived in the laboratories of Dr T M. Dexter,[24] was obtained from the laboratory of Dr Peter Quesenberry. The FDC-P1 cells were grown in RPMI 1640 medium (GIBCO) with 10% fetal bovine serum and 25% conditioned medium (CM) from the WEHI-3B mouse myelomonocytic leukemic cell line (obtained from Dr Peter Quesenberry), as described by Dexter et al.[24] The FDC-P1 cells (10^6 cells in 5 mL of medium) were added to 60 mm tissue culture dishes that contained no other cells, or a pre-established (4-week-old) adherent stromal layer from long-term bone marrow cultures of +/+ cells that had been irradiated with 9 Gy, unirradiated Sl/Sl^d stroma, or Sl/Sl^d stroma that had been irradiated with 9, 50, or 200 Gy In all cases the FDC-P1 cells were added within two hours of the time of irradiation of the stromal layers. In each of these experimental groups the FDC-P1 cells were added to four culture dishes in the absence of any WEHI-3B CM and four additional culture dishes in the presence of 25% WEHI-3B CM. The FDC-P1 cell growth stimulating or inhibiting activity of each type of adherent stromal layer was determined by counting the number of FDC-P1 cells suspended in the supernatant medium of each culture dish after four days. Similar studies were conducted to determine if CM (used in a final concentration of 50% vol/vol) obtained 2 to 3 weeks after radiation of the long-term marrow cultures, rather than the intact irradiated stroma itself, contained stimulators, or inhibitors of FDC-P1 cell growth.

RESULTS

Growth of +/+ and Sl/Sl^d mouse bone marrow cells in long-term cultures. Cumulative production of total nonadherent hemopoietic cells and hemopoietic progenitor cells in long-term cultures of WCB6F1-(+/+) and congenic WCB6F1-(Sl/Sl^d) bone marrow is shown in Figs 1 and 2, respectively. Over a 10-week period of time, Sl/Sl^d cultures produced <10% of the number of cells produced in +/+ cultures. Production of CFU-GM by Sl/Sl^d cultures also was <10% of the production in +/+ cultures; and there were almost no detectable CFU-GEMM, CFU-M, or BFU-E produced in the Sl/Sl^d cultures.

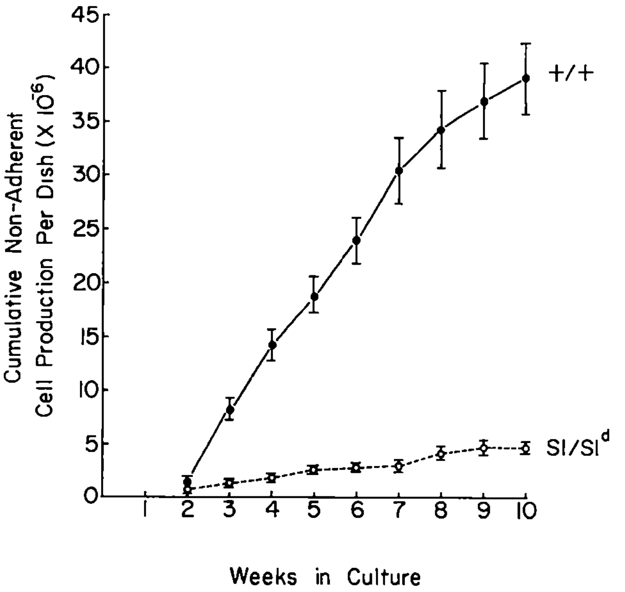

Fig 1. Cumulative hemopoietic cell production in normal (+/+) and Sl/Sl^d mouse long-term bone marrow cell cultures. Mean ± SEM for 20 separate experiments with 3 to 16 culture dishes per experiment.

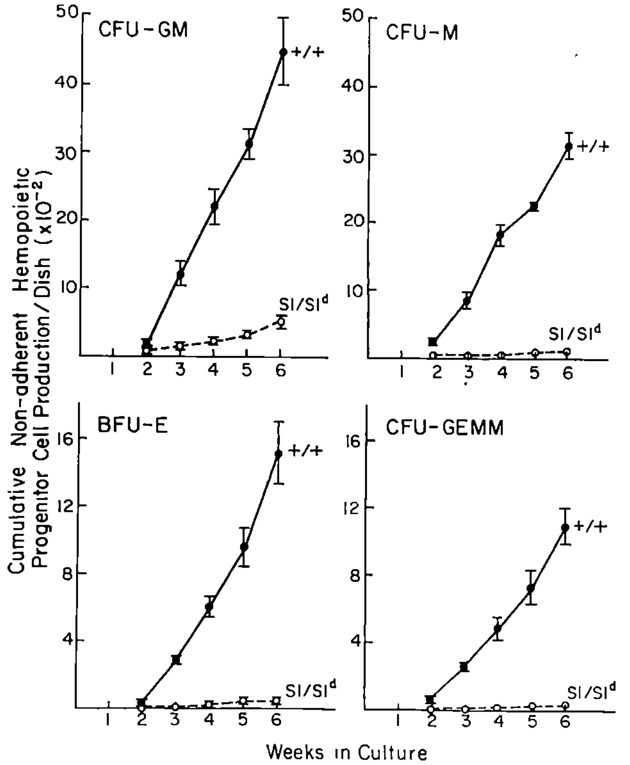

Fig 2. Cumulative production of hemopoietic progenitor cells in normal (+/+) and Sl/Sl^d mouse long-term bone marrow cultures. Mean ± SEM for eight separate experiments with three to 16 culture dishes per experiment and triplicate progenitor cell assays.

Inhibition of normal hemopoietic cell growth in vitro by the stroma of Sl/Sl^d mouse long-term bone marrow cultures. Several separate approaches were taken to determine whether the lack of hemopoiesis in Sl/Sl^d mouse long-term cultures was simply a result of lack of a necessary supportive or stimulatory material or an active inhibition of hemopoietic cell proliferation and/or differentiation by the Sl/Sl^d microenvironment. In the first set of experiments we initiated the long-term marrow cultures with mixtures of 16 x 10^6 +/+ cells along with 0, 4 x 10^6, 8 x 10^6, or 12.5 x 10^6 Sl/Sl^d cells. The results of these studies are shown in Fig 3. There was a Sl/Sl^d cell concentration–dependent inhibition of hemopoiesis by the +/+ cells inoculated into each set of cultures, with 4 x 10^6 and 8 x 10^6 Sl/Sl^d cells causing about a 35% decrease and 12.5 x 10^6 Sl/Sl^d cells causing about a 75% decrease in hemopoietic cell production over a 10-week period of culture. In fact, in the latter group there was practically no active hemopoietic cell production detected after the fifth week. For reference purposes the growth curves for +/+ cells or Sl/Sl^d cells alone cultured at the same time as the cell mixtures also are shown in Fig 3 and are virtually identical to the results for all of the other experiments shown in Fig 1. In addition, to rule out the possibility that the inhibitory effect was simply a result of culturing too many cells in each dish, we cultured 28.5 x 10^6 +/+ cells per dish (to compare with the maximum number of cells cultured in the mixture of 16 x 10^6 +/+ cells and 12.5 x 10^6 Sl/Sl^d cells). In these cultures the hemopoietic cell production was greater than that observed in cultures of 16 x 10^6 +/+ cells (cumulative production of 10.7 x 10^6 non adherent nucleated cells through week 4, 22.1 x 10^6 cells through week 6, and 41.8 x 10^6 cells through week 8). Thus, Sl/Sl^d cells added at the time of the initiation of the cultures had a true inhibitory effect on the establishment of normal long-term hemopoietic cell growth.

In the next set of studies we cultured Sl/Sl^d marrow cells for 4 weeks to permit a nearly confluent adherent stromal layer to form in the culture dishes. We then compared the growth of +/+ marrow cells added to fresh uncoated culture dishes with that of +/+ cells added to culture dishes containing a pre-established Sl/Sl^d adherent stromal layer. As shown in Fig 4, in four separate experiments in which the results were virtually identical, the Sl/Sl^d stroma caused a 68% inhibition of hemopoietic cell production over a 6-week time period. It is of further interest to note that when the Sl/Sl^d stroma was irradiated before inoculation of the fresh +/+ cells, 9 Gy had no effect, 50 Gy reduced the degree of inhibition of +/+ cell growth to 38%, and 200 Gy decreased the inhibition to 19% below control levels of hemopoietic cell production.

We wish to determine whether the inhibitory effect of Sl/Sl^d stroma was a direct local effect of the stroma or was secondary to an inhibitory material released into medium by

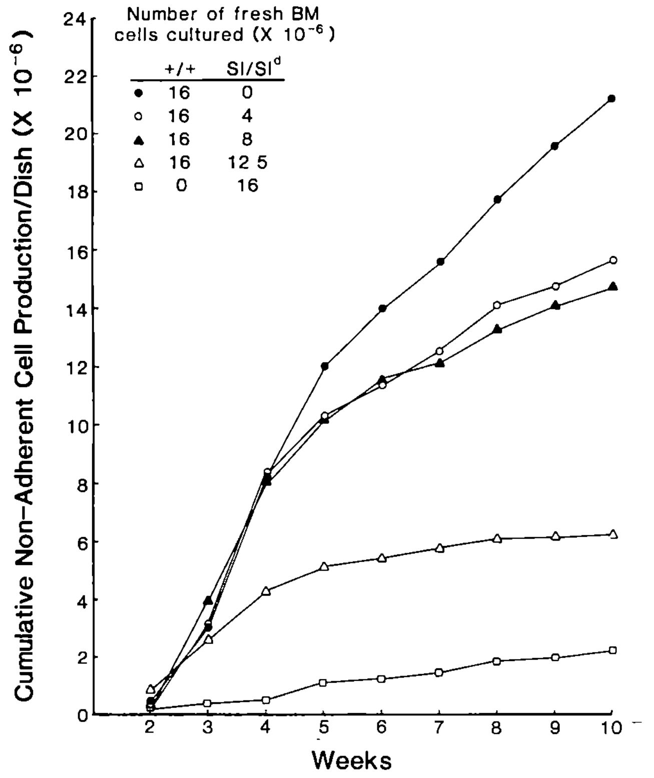

Fig 3. Cumulative production of hemopoietic cells in long-term cultures of mixtures of +/+ and Sl/Sl^d mouse bone marrow cells. The cultures were initiated with the indicated number of fresh +/+ and/or Sl/Sl^d mouse bone marrow cells. Each point represents the mean value for four culture dishes in one experiment. Variation between dishes in a given group at each time point does not exceed 15%.

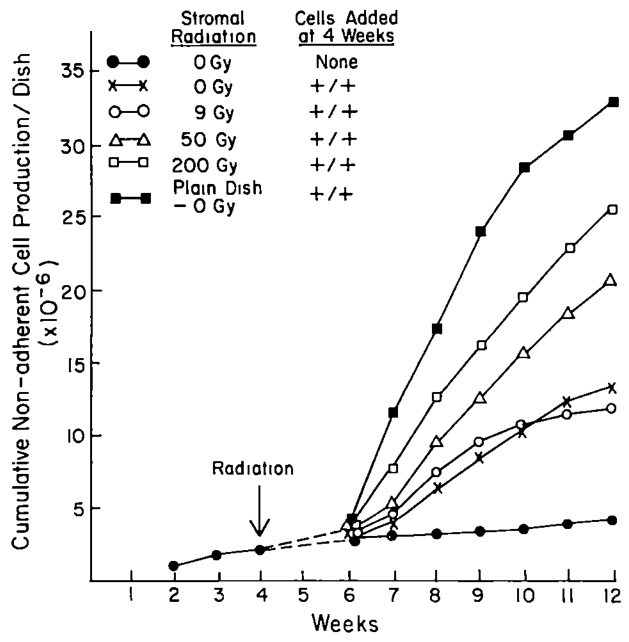

Fig 4. Effect of unirradiated and irradiated adherent stromal layers from long-term cultures of Sl/Sl^d mouse bone marrow on the long-term growth of fresh normal (+/+) mouse bone marrow cells. See the Materials and Methods section for a detailed description of the design for these experiments. Each point is the mean of four separate experiments, each with four culture dishes in every experimental group.

the stromal cells. The spent (or conditioned) media from the following culture groups were collected at weeks 1, 2, and 3 after the day of irradiation and stored at −70 °C until needed: 9 Gy irradiated +/+ cultures, unirradiated Sl/Sl^d cultures, and Sl/Sl^d cultures irradiated with 9, 50, or 200 Gy. Fresh long-term cultures of +/+ bone marrow cells were established using 100% fresh medium (as a control) or 60% fresh medium and 40% CM from one of the above groups. With each weekly change of culture medium the same combination of fresh medium and CM was added that was used to initiate the cultures. There never was any inhibitory effect of any of these CM preparations on +/+ hemopoiesis.

Finally, we wanted to confirm our findings of a direct inhibitory effect of Sl/Sl^d stroma on hemopoietic cell growth using an independent assay system. Therefore, we used the hemopoietic growth factor–dependent cell line, FDC-P1, in the presence and absence of hemopoietic growth factor–containing WEHI-3B CM. These cells (10^6 dish) were added to culture dishes that were empty or that contained pre-established, unirradiated, or irradiated Sl/Sl^d or +/+ adherent stromal layers, and FDC-P1 cells were counted after four days. The results are shown in Fig 5. None of the pre-established marrow adherent stromal layers supported FDC-P1 cell proliferation in the absence of WEHI-3B CM. Unirradiated Sl/Sl^d stroma inhibited WEHI-3B CM-dependent growth of FDC-P1 cells by 78%. Prior irradiation of the Sl/Sl^d stroma with 9 to 200 Gy eliminated the inhibition of FDC-P1 cell growth. Conditioned media from +/+ or Sl/Sl^d long-term cultures that were unirradiated or that had been irradiated with 9, 50, or 200 Gy 2 to 3 weeks previously had no inhibitory effect on WEHI-3B CM-mediated stimulation of FDC-P1 cell growth, which is similar to the observed lack of inhibition of long-term +/+ marrow cell growth by these CM preparations.

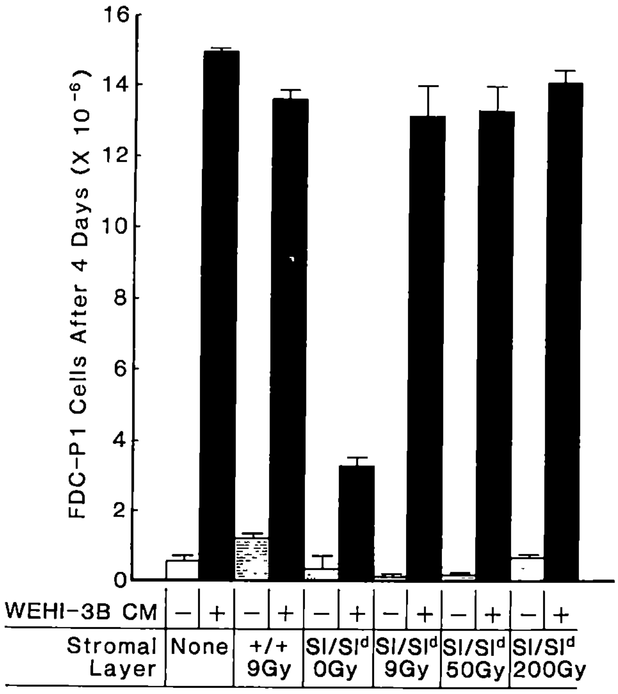

Fig 5. Effect of normal (+/+) and Sl/Sl^d mouse bone marrow adherent stromal layers on the proliferation of FDC-P1 cells. Each culture dish was inoculated with 10^6 FDC-P1 cells in the absence (light cross-hatched bars) or presence (dark solid bars) of 25% WEHI-3B-conditioned medium. The type of pre-established stromal layer and the dose of x-irradiation with which the stromal layer in the cultures was treated immediately prior to addition of the FDC-P1 cells is indicated directly below the bars. The values shown represent the mean ± SEM of the number of FDC-P1 cells recovered after four days from four separate cultures.

DISCUSSION

The functional defect of hemopoiesis in Sl/Sl^d mice in vivo has been studied extensively.[1-13] These studies have demonstrated conclusively that the abnormalities of hemopoietic cell production in this mutant mouse strain are a result of a defective hemopoietic microenvironment. Although the pathophysiologic, biochemical, and molecular genetic bases for the defect(s) remain unknown, it has been assumed on the basis of considerable indirect evidence that the Sl/Sl^d mouse hemopoietic microenvironment lacks a cell type or a secreted product of stromal cells that is essential for supporting hemopoiesis. The success of ectopically implanted spleens and bones in correcting the hemopoietic defect appears to rule out at least a circulating, widely disseminated inhibitor of hemopoiesis in Sl/Sl^d mice. However, no studies have been done that could rule out a local microenvironmental inhibitory effect on hemopoiesis.

Similarly, Dexter and Moore[15] demonstrated quite clearly that the Sl/Sl^d microenvironmental defect could be reproduced in vitro in long-term bone marrow cultures In the same set of experiments they used bone marrow cells from W/W^v mice, which have an apparently normal microenvironment, but which have defective hemopoietic progenitor cells. A pre-established W/W^v adherent stromal layer was able to support normal hemopoiesis by freshly inoculated Sl/Sl^d marrow cells. In addition, although hemopoietic cell production was significantly lower in W/W^v cells than that in normal cultures or cultures of Sl/Sl^d cells added to W/W^v stroma, hemopoiesis appeared to be diminished even further in cultures of W/W^v cells inoculated over pre-established Sl/Sl^d stromal layers. Although this might suggest the possibility that the Sl/Sl^d stroma was inhibitory to the growth of already defective hemopoietic progenitors, only studies of normal cells inoculated on Sl/Sl^d stromal layers could resolve this issue.

The present studies have confirmed that hemopoiesis is severely defective in long-term cultures of Sl/Sl^d mice. Other reports from our laboratory[14,25] have demonstrated that irradiated (9 to 50 Gy) normal stromal layers supported nearly normal hemopoietic cell growth by freshly inoculated Sl/Sl^d bone marrow cells. One important new observation in the present studies is that Sl/Sl^d bone marrow stroma not only does not support but actively inhibits the proliferation and/or differentiation of normal (+/+) hemopoietic progenitors; and this is the result of a local inhibitory factor, either cell membrane associated or extracellular matrix associated, which does not appear to be released in an active form into the supernatant medium from these cultures. These findings were confirmed in independent studies of the inhibitory effect of Sl/Sl^d stroma, but not +/+ stroma, on the hemopoietic growth factor–dependent proliferation of FDC-P1 cells, which provides a potentially important new model system for studying stromal effects on hemopoietic progenitor cell growth. Thus, it seems to be quite clear that there is a local inhibitor of hemopoiesis in the bone marrow stroma of Sl/Sl^d mice. Although neither we nor other groups[15,16,18,19] have been able to detect morphologic differences in stromal cell types between +/+ and Sl/Sl^d long-term marrow cultures, we cannot rule out the possibility that this inhibitory effect of Sl/Sl^d marrow stroma on normal hemopoiesis results from an as yet unidentified cell type that is present in Sl/Sl^d but not in +/+ marrow stroma.

A second important finding of the present study is that the inhibitory effect of Sl/Sl^d stroma on normal hemopoiesis and on FDC-P1 cell proliferation can be abrogated by irradiating the Sl/Sl^d stroma even with doses of x-irradiation that do not cause loss of stromal cells or morphologic changes in the stroma that are detectable by phase contrast microscopy.[25] We do not know the explanation for these observations. However, several potential mechanisms need to be considered. One possibility is that the radiation could stimulate production or release or inhibit metabolism of a soluble stimulator of hemopoiesis by the Sl/Sl^d stromal cells. This seems to be very unlikely for several reasons: (1) the nearly 50% general decrease in protein synthesis noted in cultures irradiated with as little as 9 Gy[25]; (2) the lack of any differential enhancing effect of spent media from unirradiated versus 9, 50, or 200 Gy irradiated Sl/Sl^d cultures on hemopoiesis in fresh +/+ long-term bone marrow cultures (unpublished data); and (3) the lack of inhibitory effect of spent medium from unirradiated Sl/Sl^d cultures on +/+ long-term culture growth. Another possibility is that irradiation of the stroma could enhance metabolism of a soluble inhibitor of normal hemopoiesis. Our studies that showed no inhibitory effect of spent media from unirradiated Sl/Sl^d cultures suggest that this is not the explanation for our observations. Our results seem to indicate that the hemopoietic inhibitory activity of Sl/Sl^d stroma is either cell surface bound or extracellular matrix bound. Thus, radiation of the Sl/Sl^d stroma must result in one of the following: (1) loss of a minor population of inhibitory stromal cells, which we are unable to detect morphologically; (2) cessation of production of an inhibitory cell surface or extracellular matrix substance; or (3) increased metabolism of the inhibitory, but not of necessary supportive, stroma-associated components. Although we cannot rule out the first possibility, we could not detect stromal cell loss. Furthermore, it would be difficult to implicate any quantitatively minor, directly inhibitory cell population that would be selectively lost, since the inhibitory effect is restricted to the stromal layer itself (ie, not secreted into the medium), but the inhibition of hemopoiesis is virtually universal, with essentially no active hemopoietic cell growth on the entire area of the adherent stromal layer of the unirradiated Sl/Sl^d long-term marrow cultures. Thus, the radiation most likely eliminates production or release of an inhibitory cell surface material that is present on most cells in the stroma or an inhibitory extracellular matrix component that is widespread throughout the adherent stromal layer of Sl/Sl^d marrow cultures. It is tempting to speculate that normal (+/+) stromal cells produce a hemopoietic-supportive counterpart of the inhibitory Sl/Sl^d stromal protein, but that the defective Sl/Sl^d protein competes successfully against the normal (+/+) derived protein for binding sites, and thus inhibits hemopoietic cell production by fresh +/+ cells placed in contact with unirradiated Sl/Sl^d marrow stroma. Obviously, such a hypothesis can be tested only by finding differences in the stromal-associated proteins of the normal and mutant mice, purifying the proteins of interest, and studying the binding properties of such proteins, if they exist. We have reported the finding of definite differences in the bone/bone marrow extracellular matrix proteins of +/+ and Sl/Sl^d mice.[18,19] Nonetheless, a great deal more work needs to be done before there could be convincing evidence of such a critical extracellular matrix protein abnormality that is responsible for the microenvironmental defect(s) of Sl/Sl^d mice.

REFERENCES

1. Trentin JJ: Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironment (HIM). Am J Pathol 65:621, 1971

2. Wolf NS. Dissecting the hematopoietic microenvironment. I Stem cell lodgment and commitment, and the proliferation and differentiation of erythropoietic descendants in the Sl/Sl^d mouse. Cell Tissue Kinet 7:89, 1974

3. Bernstein SE, Russell ES, Keighley G· Two hereditary mouse anemias (Sl/Sl^d and W/W^v) deficient in response to erythropoietin. Ann NY Acad Sci 149:475, 1968

4. Bernstein SE: Tissue transplantation as an analytic and therapeutic tool in hereditary anemias. Am J Surg 119:448, 1970

5. Russell ES: Hereditary anemias of the mouse: A review for geneticists. Adv Genetics 20:357, 1979

6 Harrison DE: Use of genetic anemias in mice as tools for haematological research. Clin Haematol 8:239, 1979

7. Russell ES· Genetic aspects of implantation of blood-forming tissue Fed Proc 19:573, 1960

8. McCulloch EA, Siminovitch L, Till JE, Russell ES, Bernstein SE: The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl/Sl^d. Blood 26·399, 1965

9. Altus MS, Bernstein SE, Russell ES, Carsten AL, Upton AC· Defect extrinsic to stem cells in spleens of anemic mice. Proc Soc Exp Biol Med 138:985, 1971

10. Trentin JJ, McGarry MP, Jenkins VK, Gallagher MT, Spiers RS, Wolf NS· Role of inductive microenvironments on hemopoietic (and lymphoid?) differentiation and role of thymic cells in the eosinophilic granulocyte response to antigen, in Lindahl-Kiessling K, Alm G, Hanna MG (eds)· Morphological and Functional Aspects of Immunity. New York, Plenum Press, 1971, p 289

11. Fried W, Chamberlin W, Knospe WH, Husseini S, Trobaugh FE Jr: Studies on the defective haematopoietic microenvironment of Sl/Sl^d mice. Br J Haematol 24:643, 1973

12. Ruscetti FW, Boggs DR, Torok BJ, Boggs SS: Reduced blood and marrow neutrophils and granulocytic colony forming cells in Sl/Sl^d mice. Proc Soc Exp Biol Med 152:398, 1976

13. Ebbe S, Phalen E, Stohlman F Jr· Abnormalities of megakaryocytes in Sl/Sl^d mice. Blood 42:865, 1973

14. Zuckerman KS, Rhodes RK, Sparks B, Chenier B· Correction of the hemopoietic microenvironmental defect of Sl/Sl^d mice by relatively radiation-resistant elements in normal bone marrow stroma. Exp Hematol 13:413, 1985 (abstr)

15. Dexter TM, Moore MAS: In vitro duplication and "cure" of hematopoietic defects in genetically anaemic mice Nature 269.412, 1977

16. Wiktor-Jedrzejczak W, Ptasznik A, Ahmed A, Szczylik C: Adherent cell growth from murine bone marrow in liquid cultures Inbred strain variability and effects of mutations affecting hemopoiesis. Exp Hematol 11:63, 1983

17. Keller GM, Phillips RA: Maintenance of hemopoiesis in long-term bone marrow cultures from Sl/Sl^d and W/W^v mice. Exp Hematol 12:822, 1984

18 Zuckerman KS, Rhodes RK, Sparks B, Chenier B, Gay R, Denys F: Characterization of the hemopoietic stroma of Sl/Sl^d mouse bone marrow in vitro. Clin Res 33.358A, 1985 (abstr)

19. Zuckerman KS, Prince CW, Gay R, Denys F: The role of extracellular matrix in the regulation of hematopoiesis, in Zanjani ED, Tavassoli M, Ascensao J (eds): Humoral and Cellular Regulation of Erythropoiesis. New York, Spectrum Publications, 1986 (in press)

20. Zuckerman KS, Wicha MS: Extracellular matrix production by the adherent cells of long-term murine bone marrow cell cultures. Blood 61:540, 1983

21 Zuckerman KS, Rhodes RK, Goodrum DD, Patel VR, Sparks B, Wells J, Wicha MS, Mayo LA: Inhibition of collagen deposition in the extracellular matrix prevents the establishment of a stroma supportive of hematopoiesis in long term murine bone marrow cultures. J Clin Invest 75.970, 1985

22. Dexter TM, Allen TD, Lajtha LG: Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:355, 1977

23. Dexter TM, Testa NG: Differentiation and proliferation of hemopoietic cells in culture. Methods Cell Biol 14:387, 1976

24. Dexter TM, Garland T, Scott D, Scolnick E, Metcalf D. Growth of factor-dependent hematopoietic precursor cell lines. J Exp Med 152:1036, 1980

25. Zuckerman KS, Prince CW, Rhodes RK, Ribadeneira M: Resistance of the stromal cells in murine long term bone marrow cell cultures to damage by ionizing radiation. Exp Hematol 13:372, 1985 (abstr)

The Heterogeneity of Type IIA von Willebrand's Disease: Studies With Protease Inhibitors

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The absence of large von Willebrand factor (vWF) multimers from plasma is a characteristic of Type IIA von Willebrand's disease (vWD) and is thought to contribute to the clinical expression of this disorder. Recently, three IIA patients have been reported in whom intermediate and large multimers could be restored if blood were collected in 5 mm EDTA, 6 mmol/L N-ethylmaleimide, and 1 mmol/L leupeptin. This suggested that absence of large multimers resulted from in vitro proteolysis. We have now collected blood from ten Type IIA vWD patients in these inhibitors but were not able to detect large multimers in the plasma of any of them. In addition, intermediate-sized multimers were reduced or completely absent in all. The inclusion of inhibitors in the citrate anticoagulant, as compared to citrate alone, was found to increase the relative proportion of intermediate multimers in some patients but had no effect in others, and in none did it restore large multimers to plasma. The results with platelet vWF were more varied. Four patients showed an absence or decrease of large multimers, whereas in seven patients large multimers were present. When compared with citrate anticoagulant alone, the inclusion of inhibitors in the anticoagulant had little or no effect on the platelet multimeric pattern. 1-Deamino-8-D-Arginine Vasopressin (DDAVP) was administered to six patients from five families. Two patients from one family showed complete correction and a third patient showed almost complete correction of her bleeding time. Two patients showed minimal correction and one showed no detectable correction. An increase in multimer size after DDAVP tended to be associated with correction of the bleeding time. However, in no case did the largest multimers appear in plasma even in patients with complete bleeding time correction. The presence or absence of inhibitors in the anticoagulant had little or no effect on the multimeric pattern after DDAVP. These results indicate that Type IIA vWD is a heterogeneous disorder in which absence of largest and intermediate multimers is an in vivo phenomenon.

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THE initial descriptions of Type IIA von Willebrand's disease (vWD) demonstrated absence of large von Willebrand factor (vWF) multimers from plasma.[1,2] Large multimers were also absent from platelets in some patients,[1] but this was not a universal finding.[2] The use of high resolution agarose gels has since revealed that each multimer consists of three or more bands. In IIA vWD the minor bands are accentuated, particularly the fastest moving band in each multimer group.[3] Ristocetin-induced platelet aggregation is decreased to absent, a feature distinguishing Type IIA from Type IIB vWD. In the latter disorder, absence of large multimers is associated with hyper-responsiveness to ristocetin.[1,4] Additional subtypes have been described that lack large multimers and have a decreased response to ristocetin. However, these have a multimer banding pattern that is distinct from that seen in IIA vWD and normal plasma.[5-8] Heterogeneity within Type IIA has been suggested by variability in the response to 1-Deamino-8-D-Arginine Vasopressin (DDAVP), both in regard to the presence or absence of bleeding time correction and in the appearance or non-appearance of large multimers in plasma.[9-11]

Recently, three Type IIA patients were reported in whom large multimers of vWF were largely restored to plasma and platelets when blood was collected in the presence of protease inhibitors (leupeptin, N-ethylmaleimide (NEM), and EDTA).[12] This suggested that absence of large multimers was the result of in vitro proteolysis, possibly by the platelet calcium-activated protease.[13]

In order to evaluate the role of in vitro proteolysis in producing the multimeric pattern of IIA vWD, we have collected blood from ten individuals with this disorder in 1 mmol/L leupeptin, 6 mmol/L NEM, and 5 mmol/L EDTA. The effect of DDAVP on the bleeding time and the multimeric pattern was also evaluated in six of them. The results indicated that absence of large multimers in this group of Type IIA patients was not the result of in vitro proteolysis and that the response to DDAVP in this subtype is varied.

MATERIALS AND METHODS

Seven female and three male patients from seven different kindreds, aged between 3 and 49 years, were studied. All met the following criteria for Type IIA vWD: they had lifelong bleeding diathesis, their bleeding time was prolonged beyond 20 minutes in all except one patient (15 minutes), ristocetin-induced platelet agglutination was reduced or absent, and large multimers were absent from plasma of blood collected in acid citrate dextrose (ACD) anticoagulant. Pertinent laboratory data are summarized in Table 1.

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Table 1. Laboratory Values of IIA Patients

	Sex	BT	BT After DDAVP	VIII:C (U/dL)	vWF:Ag (U/dL)	vWF:RCo (U/dL)	RIPA
P1	F	15		96	152	6.5	D+
{P2	F	>30	9.5	100	50	<1	D+
{P3	F	>30		50	60	<1	D+
P4	F	>30	16	50	40	<1	A
P5	F	>30		40	45	2	A
{P6	M	>30	21	33	26	5	A
{P7	M	>20		29	21	4	A
{P8	F	>20	4.5	35	46	22	D++
{P9	M	>30	7	30	50	22	D++
P10	F	>20	>20	60	54	<1	A
Normal		<8.5		>50	>45	>45	P+

D+, absent at 1.5 mg/mL of ristocetin, decreased but present at 2 mg/mL of ristocetin; D++, decreased but present at 1.5 mg/mL; A, absent (patients 6–10 were tested at 1.5 mg/mL only); P+, present; {, kindred.

Blood for multimeric studies and von Willebrand factor antigen (vWF:Ag) quantitation was collected into $^1/_{10}$ volume of 3.8% sodium citrate or $^1/_6$ volume of ACD pH 4.5, containing sufficient EDTA, leupeptin, and N-ethylamaleimide (from Sigma, St. Louis, Mo), to give final concentrations in blood of 5 mmol/L, 1 mmol/L, and 6 mmol/L, respectively. One aliquot was rapidly spun down at 3,500 rpm, at 24 °C, for 20 minutes and the PPP collected. Another aliquot was used for preparing platelet lysates as described elsewhere[14] but including the above mentioned inhibitors throughout the entire process. All the samples were used immediately or frozen at −70 °C until used. In some instances, blood was collected in citrate or ACD alone for comparison.

Blood was also collected and added to citrate or ACD with no inhibitors for Factor VIII procoagulant activity (VIII:C) assay. Ristocetin-induced platelet agglutination (RIPA) assays were carried out as described previously.[8] vWF:Ag was measured by electroimmunoassay (EIA).[15] All quantitative assays were referred to average normal plasma (a pool of at least 20 normal volunteers), which was defined as containing 100 arbitrary units per deciliter (U/dL).

The multimeric composition of vWF was analyzed by thin-layer agarose electrophoresis in the presence of SDS, using a discontinuous buffer system.[16] vWF multimers were identified autoradiographically by reacting the gels with ^{125}I-labelled affinity purified anti-vWF rabbit antibody obtained as described.[15]

The protocol for the infusion of 1-Deamino-8-D-Arginine-Vasopressin (DDAVP; Minirin, Ferring Laboratories, Malmo, Sweden or Stimate, Armour Pharmaceutical, Tuckahoe, NY) has been previously described[9] and was approved by the appropriate institutional review boards in Spain and the United States. DDAVP was infused at a dose of 0.3 or 0.4 μg/kg with blood samples obtained and bleeding time measured (Simplate II, General Diagnostics, Anaheim, Calif) before and after the infusion.

RESULTS

The effect of inhibitors on vWF multimeric structure. Inclusion of 5 mmol/L EDTA, 6 mmol/L NEM, and 1 mmol/L leupeptin in the anticoagulant did not result in the restoration of large multimers to plasma vWF (Figs 1, 2, 3,

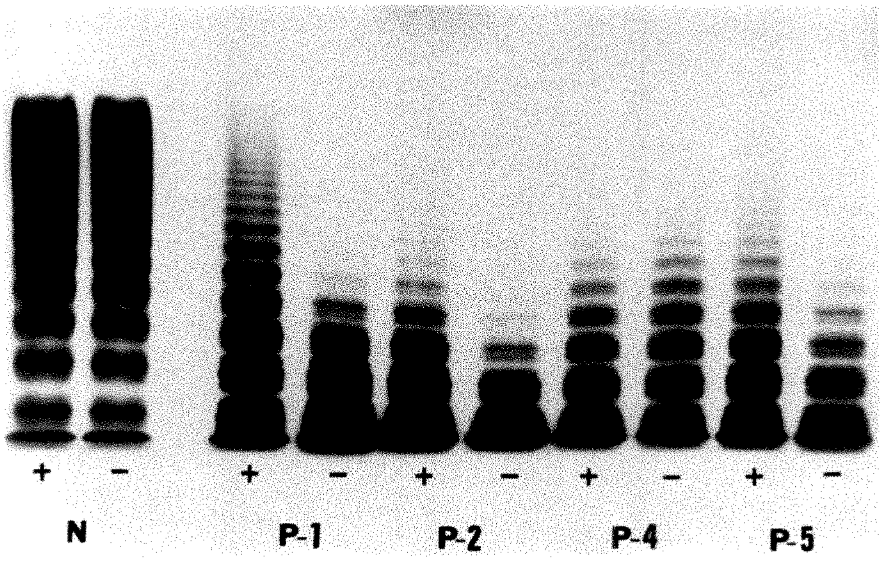

Fig 1. SDS-1.4% agarose gel electrophoresis of vWF from normal plasma (N) and vWD patient plasmas (P-1, P-2, P-4, and P-5) collected in the presence (+) of 5 mmol/L EDTA, 6 mmol/L NEM, and 1 mmol/L leupeptin or with citrate anticoagulant alone (−). Inclusion of inhibitors in P-1, P-2, and P-5 plasmas resulted in the appearance of intermediate multimers not present in their absence. However, no difference is seen in P-4 plasma and in no case were the largest multimers present in normal plasma detected in patient plasmas.

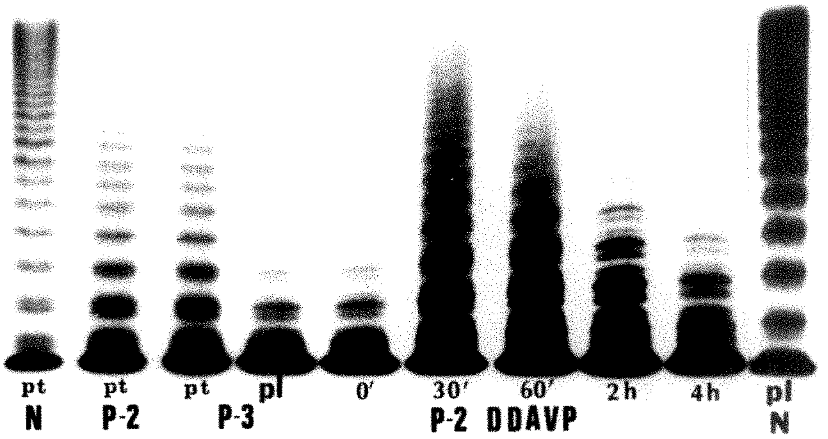

Fig 2. SDS-1.4% agarose gel electrophoresis of platelet (pt) and plasma (pl) vWF from a normal individual (N) and two patients (P-2 and P-3). Blood for all samples was collected in the presence of EDTA, NEM, and leupeptin. Plasma vWF from P-2 before and after DDAVP is also shown. The largest multimers could not be detected in the platelets of either patient. After DDAVP there was a considerable increase in intermediate multimers of P-2 but the largest multimers were not detected even when the bleeding time correction was most complete (30 and 60 minutes).

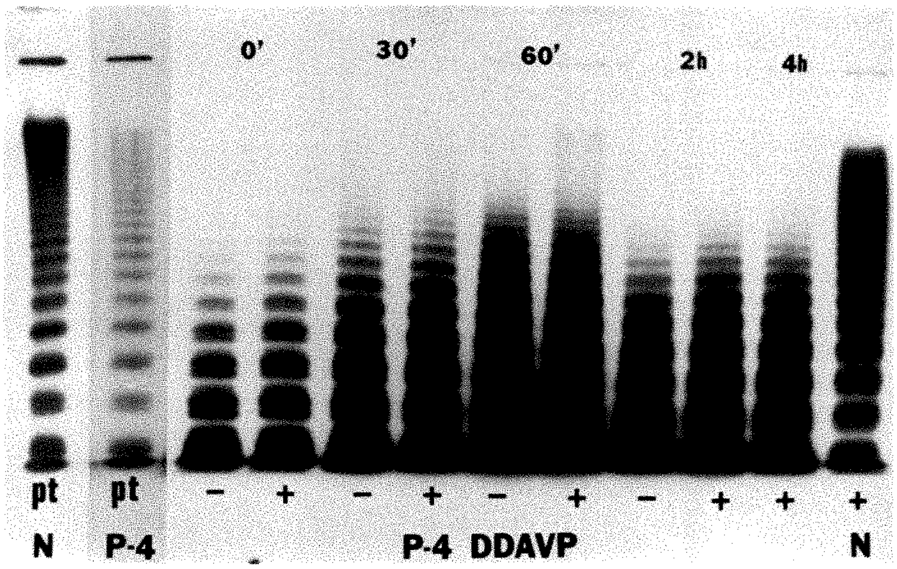

Fig 3 SDS-1.4% agarose gel of vWF from patient number 4 (P-4) and normal (N) platelets (pt) and plasma. The platelet vWF was from blood collected in the presence of EDTA, NEM, and leupeptin. Plasma vWF was from blood collected both in the absence (–) and presence (+) of inhibitors. The largest multimers were present in patient platelets but did not appear in patient plasma after DDAVP (30', 60', 2 hours, and 4 hours), and even though there was a considerable increase in the amount of vWF in plasma (see Fig 7), the presence of inhibitors had little or no effect on the multimeric pattern.

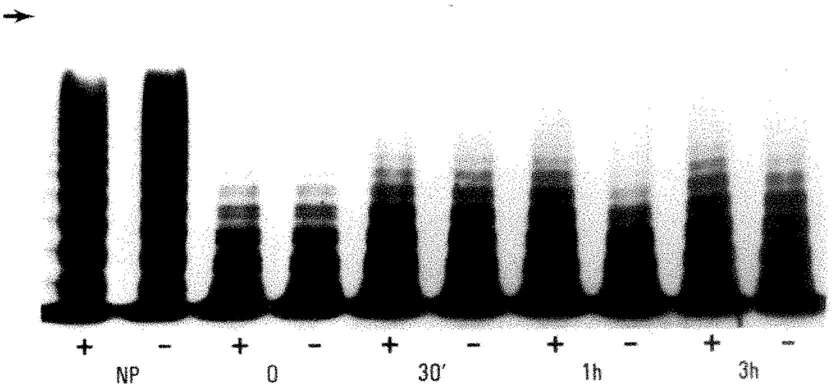

Fig 4. SDS-1.4% agarose gel of vWF in normal plasma (NP) and patient number 10 plasma before (0) and after (30', 1 hour, 2 hours) DDAVP. Blood was collected in the presence (+) or absence (–) of inhibitors. Minimal change in multimeric pattern was seen after DDAVP. The bleeding time did not shorten to less than 30 minutes. The presence of inhibitors had a discernible effect on the multimeric pattern only at one hour. Arrow indicates origin of resolving gel.

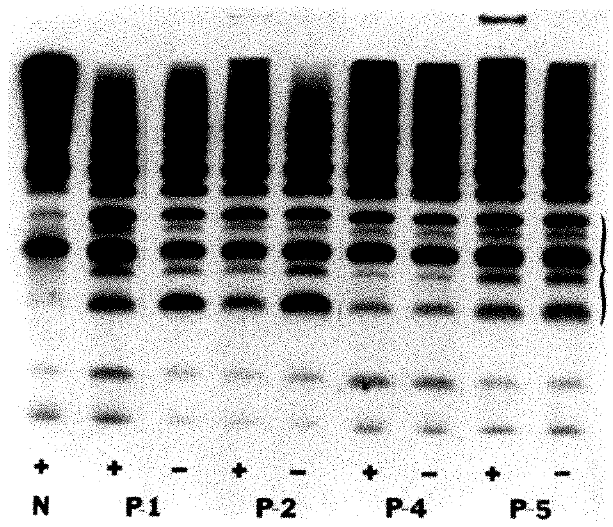

Fig 5. SDS-2.2% agarose gel electrophoresis of normal plasma (N) and patient plasmas (P-1, P-2, P-4, and P-5) collected with (+) and without (−) inhibitors. At this concentration of agarose the smallest complete multimer (bracket) is resolved into five bands including the major central band. These additional bands are greatly accentuated in the patient plasmas but are also faintly discernible in normal plasmas (see also Fig 6). Two additional minor bands are also seen at the bottom of the gel in normal and patient plasma. The inclusion of EDTA, NEM, and leupeptin (+) had little or no effect on the intensity of any of these bands. At this concentration of agarose large multimers were poorly resolved and their absence from patient plasma was often not clear.

4). Though intermediate multimers were often present, they were always decreased relative to small multimers when compared with the relative proportions in normal plasma.

The multimeric structure was compared in plasmas from five patients in which blood was collected with and without inhibitors. In two patients (#4 and #10) there was slight or no difference in the resting state or after DDAVP. Patient #4 is shown in Figs 1, 3, 5, and 6, patient #10 in Fig 4. In three additional patients, numbers 1, 2, and 5, significantly more intermediate-sized multimers were present when inhibitors were included in the anticoagulant than in the absence of inhibitors (Fig 1). However, in no case was the proportion of intermediate multimers the same as normal, or were large multimers restored.

The multimeric composition of platelet vWF was more variable. Intermediate multimers were present in all patients. However, in patients #2 and #3 large multimers were not detectable (Fig 2). In the remaining patients large multimers were present (Figs 3 and 6) whether or not inhibitors were used.

Increasing the concentration of agarose in the running gel from 1.4% to 2.2% resolved the individual multimers into three or more components. In normal plasma the predominant central band was seen with less intense bands above and below. These "satellite" bands were accentuated in the plasma of the IIA patients, especially those that moved faster than the central band. However, little or no difference in the intensity of these bands was seen in the presence or absence of inhibitors and no bands were present that could not be discerned, at least faintly, in normal plasma.

The higher resolution 2.2% gels were misleading for evaluation of the presence or absence of large or intermediate multimers. Thus, patients #4 and #5 showed absence of large and intermediate multimers from plasma with the 1.4% gel (Fig 1). However, when examined in 2.2% gels, absence of these multimers was not apparent (Fig 5).

Response to DDAVP. The bleeding time response to DDAVP varied from undetectable to complete correction. Patient #10 had a bleeding time >20 minutes before and after DDAVP. Only the smaller multimers were present in her plasma and only minimal changes occurred after DDAVP (Fig 4). With the exception of the one hour time point, no significant difference could be discerned in the multimeric pattern whether or not inhibitors were used in the anticoagulant and even at this time the difference was modest (Fig 4).

Patient #4 showed a modest change in bleeding time after DDAVP, dropping from 26 to 15 minutes (Fig 7). Patient #6 (not shown) showed a drop in bleeding time from >30

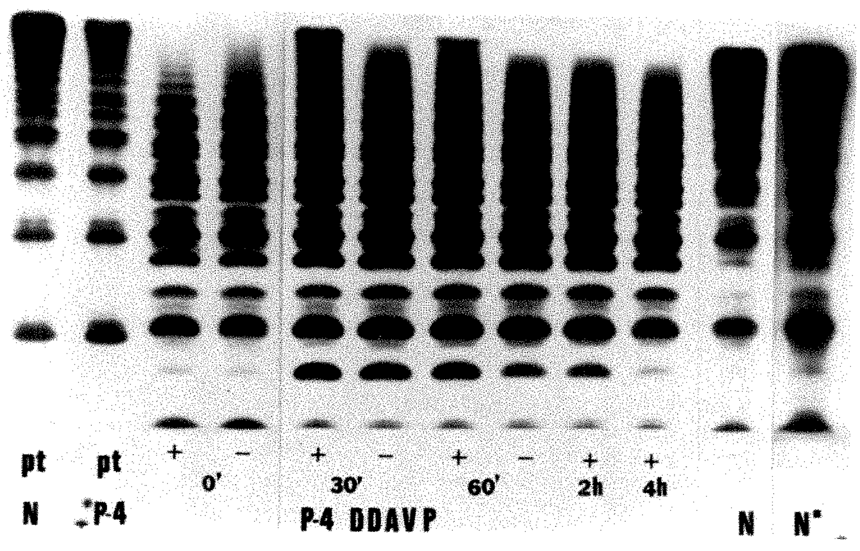

Fig 6. SDS-2.2% agarose gel of vWF from plasma of patient #4 before (0') and after (30', 60', 2 hours, 4 hours) DDAVP collected in the presence (+) or absence (−) of inhibitors. vWF from normal plasma (N) is shown for comparison. The two left-hand lanes show platelet vWF (pt) from normal plasma and patient #4. The autoradiograph exposure time for normal plasma was extended (N*) to better show the minor bands in each multimer group. It can be seen that the fastest moving band in the first multimer group was particularly accentuated in the patient plasma, although there was no evident difference in this band whether or not inhibitors were used.

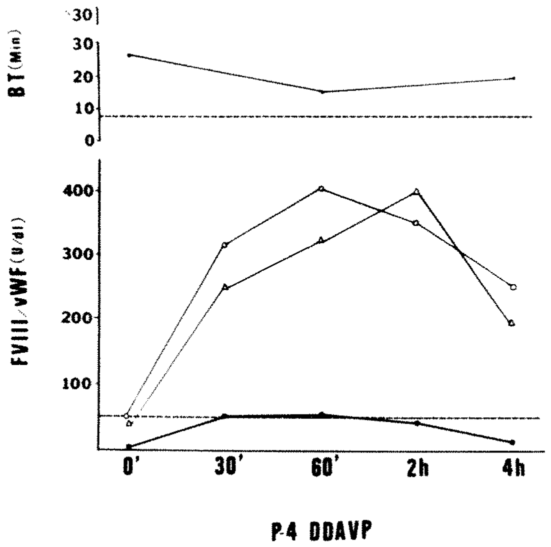

Fig 7. Response of patient #4 (P-4) to DDAVP. Only minimal correction of the bleeding time was seen. △—△, vWF:Ag; ○—○, VIII:C; ●—●, vWF:RCo.

minutes to 21 minutes. Both individuals had modest increases in the size of circulating multimers (Fig 3). The presence or absence of inhibitors had minimal effect on the multimeric pattern in regard to the size of multimers (Fig 3) or the banding pattern as determined with the higher resolution gels (Fig 6). However, an increase in the relative concentration of the fastest migrating band in the smallest multimer was noted between 30 minutes and two hours after DDAVP, even in the presence of inhibitors (Fig 6).

Almost complete correction of the bleeding time (>30 minutes to 9.5 minutes) was seen in patient #2 (Fig 8) and complete correction occurred after DDAVP in patients #8 and #9 (>20 minutes to 4.5 minutes and >30 minutes to 7 minutes, respectively). Though these patients showed a somewhat greater increase in the size of plasma multimers than the other patients (Fig 2), in no case were the largest multimers restored. It is of interest that the patients who showed complete correction of the bleeding time were from the same family and showed the highest resting ristocetin cofactor levels (22 U/dL in both cases) and reached normal levels after DDAVP (102 and 82 U/dL, respectively).

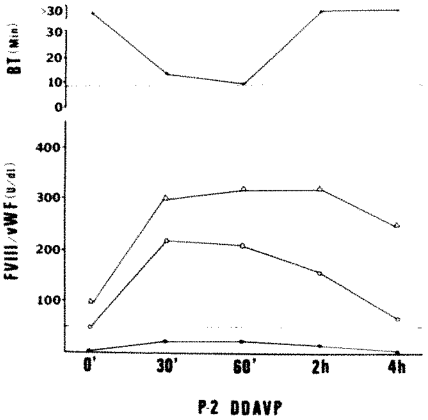

Fig 8. Response of patient #2 (P-2) to DDAVP. Almost complete correction of the bleeding time was seen. △—△, vWF:Ag; ○—○, VIII:C; ●—●, vWF:RCo.

DISCUSSION

These studies extend previous observations showing absence of large and intermediate forms of vWF from plasma of patients with Type IIA vWD. They also emphasize the heterogeneous nature of the disorder. The degree to which intermediate multimers were absent from plasma varied from patient to patient as did the presence or absence of large forms from platelets. The response to DDAVP also varied in this group from complete correction of the bleeding time to virtually no effect.

In some patients, inclusion of NEM, EDTA, and leupeptin in the anticoagulant into which blood was collected for study did result in the demonstration of some intermediate-sized multimers in plasma which were not evident in the absence of the inhibitors. In other patients, however, these inhibitors were without effect and in no case did they restore large multimers to plasma. No effect on platelet vWF was discerned.

No vWF bands were present in IIA patient plasma that could not be detected in normal plasma. However, in high resolution gels it was evident that "satellite" bands were considerably more prominent in IIA vWF plasma than in normal plasma, as we have previously reported.[3] These bands were only minimally affected, or not at all, by the presence of inhibitors.

The effect of inhibitors on the plasma multimeric pattern after DDAVP was also minimal or not detectable. None of the patients studied here showed restoration of large multimers to plasma whether or not their bleeding times were restored to normal by DDAVP. We have recently reported a patient with many of the characteristics of Type IIA in whom large multimers were evident in plasma after DDAVP in the absence of protease inhibitors.[10]

The observations reported here differ from the recent reports of Gralnick and coworkers of three IIA patients in whom inclusion of similar inhibitors restored large multimers to plasma in the resting state[12] and after DDAVP.[11] These workers also detected a rapidly moving band in the absence of inhibitors that was not evident in their presence. These differences may reflect additional heterogeneity in this disease or may be in part technical. The gel system used by Gralnick and coworkers differed from that used by us and may have resolved large and intermediate multimers differently. In our system, simply raising the agarose concentration markedly decreased the ability of the gels to resolve large and intermediate sized multimers, although it improved resolution of individual components of each multimer. A single gel concentration was not adequate in our hands to evaluate both aspects of multimeric structure.

The studies of Gralnick and coworkers suggest that loss of large multimers is essentially an in vitro process resulting from an increased susceptibility of IIA vWF to proteolysis and that appeared largely correctable by the addition ex vivo of protease inhibitors.[12] In contrast, these inhibitors did not restore large multimers to the plasmas of any of the patients studied here. In addition, we have recently reported increased fragmentation of the vWF subunit from several of the IIA patients studied here even though plasma was collected and processed in the presence of the same inhibitors.[17] This evidence together with the data presented in this paper indicates that absence of large multimers in IIA vWD is the result, at least in part, of in vivo proteolysis of vWF.

ACKNOWLEDGMENT

We wish to thank Mrs Ruth Stewart and Ms Claire Jackson for their secretarial assistance.

REFERENCES

1. Ruggeri ZM, Zimmerman TS: Variant von Willebrand's disease: Characterization of two subtypes by analysis of multimeric composition of Factor VIII/von Willebrand factor in plasma and platelets. J Clin Invest 65:1318, 1980

2. Weiss HJ, Pietu G, Rabinowitz R, Girma J-P, Rogers J, Meyer D: Heterogeneous abnormalities in the multimeric structure, antigenic properties, and plasma-platelet content of factor VIII/von Willebrand factor in subtypes of classic (type I) and variant (type IIA) von Willebrand's disease. J Lab Clin Med 101:411, 1983

3. Ruggeri ZM, Zimmerman TS: The complex multimeric composition of Factor VIII/von Willebrand factor. Blood 57:1140, 1981

4. Ruggeri ZM, Pareti FI, Mannucci PM, Ciavarella N, Zimmerman TS: Heightened interaction between platelets and Factor VIII/von Willebrand Factor in a new subtype of von Willebrand's disease. N Engl J Med 302:1047, 1980

5. Ruggeri ZM, Nilsson IM, Lombardi R, Holmberg L, Zimmerman TS: Aberrant multimeric structure of von Willebrand factor in a new variant of von Willebrand's disease (type IIC). J Clin Invest 70:1124, 1982

6. Kinoshita S, Harrison J, Lazerson J, Abildgaard CF: A new variant of dominant Type II von Willebrand's disease with aberrant multimeric pattern of Factor VIII-related antigen (Type IID). Blood 63:1369, 1984

7. Mannucci PM, Lombardi R, Pareti F, Solinas S, Mazzucconi MG, Mariani G: A variant of von Willebrand's disease characterized by recessive inheritance and missing triplet structure of von Willebrand factor multimers. Blood 62:1000, 1983

8. Batlle J, Lopez Fernandez MF, Lasierra J, Fernandez Villamor A, Lopez Berges C, Lopez Borrasca A, Ruggeri ZM, Zimmerman TS: von Willebrand's disease Type IIC with different abnormalities of von Willebrand factor in the same sibship. Am J Hematol 21:177, 1986

9. Ruggeri ZM, Mannucci PM, Federici AB, Lombardi R, Zimmerman TS: Multimeric composition of Factor VIII/von Willebrand factor following administration of DDAVP: Implications for pathophysiology and therapy of von Willebrand's disease subtypes. Blood 59:1272, 1982

10. Hanna WT, Dent J, Ruggeri ZM, Zimmerman TS: DDAVP and cryoprecipitate combination in variant von Willebrand's disease. Am J Hematol 20:169, 1985

11. Gralnick HR, Williams SB, McKeown LP, Rick ME, Maisonneuve P, Jenneau C, Sultan Y: DDAVP in Type IIA von Willebrand's disease. Blood 67:465, 1986

12. Gralnick HR, Williams SB, McKeown LP, Maisonneuve P, Jenneau C, Sultan Y, Rick ME: In vitro correction of the abnormal multimeric structure of vWF in type IIA vWD. Proc Natl Acad Sci USA 82:5968, 1985

13. Kunicki TJ, Montgomery RR, Shulleck J: Cleavage of human von Willebrand factor by platelet calcium-activated protease. Blood 65:352, 1985

14. Lopez Fernandez MF, Batlle J, Ruggeri ZM, Zimmerman TS: Secretion of von Willebrand factor from platelets. Meth Enzymol (in press)

15. Zimmerman TS, Roberts JR: Factor VIII-related antigen, in Nakamura RM, Dito WR, Tucker ES III (eds): Immunoassays: Clinical Laboratory Techniques for the 1980's. New York, Alan R. Liss, 1980, pp 339–349

16. Zimmerman TS, Batlle FJ, Ruggeri ZM, Roberts JR: Multimeric analysis of Factor VIII/von Willebrand Factor in the diagnosis of von Willebrand's disease, in Nakamura RM, Dito WR, Tucker ES III (eds): Clinical Laboratory Assays: New Technology and Future Directions. New York, Masson Publishing, 1983, pp 249–263

17. Zimmerman TS, Dent JA, Ruggeri ZM, Nannini LH: Subunit composition of plasma vWF: Cleavage is present in normal individuals, increased in IIA and IIB vWD but minimal in variants with aberrant structure of individual oligomers (Types IIC, IID, and IIE). J Clin Invest 77:947, 1986

Subunit Composition of Plasma von Willebrand Factor in Patients With the Myeloproliferative Syndrome

By Ulrich Budde, Judith A. Dent, Scott D. Berkowitz, Zaverio M. Ruggeri, and Theodore S. Zimmerman

In order to evaluate the role of proteolysis in acquired von Willebrand's disease (vWD) associated with the myeloproliferative syndrome, we have determined the relative quantity of von Willebrand factor (vWF) fragments as compared with the intact 225 kDa subunit in four patients. The plasma vWF of each individual lacked large multimers; each had a prolonged bleeding time; and both platelet and leukocyte counts were elevated. Plasma was obtained from blood drawn into 1 mmol/L leupeptin, 6 mmol/L N-ethylmaleimide, and 5 mmol/L EDTA to prevent in vitro proteolysis. vWF was isolated from plasma by immunoadsorbent chromatography, reduced, subjected to SDS-5% polyacrylamide gel electrophoresis, and immunoblotted with a mixture of 55 anti-vWF monoclonal antibodies. In three patients with essential thrombocytosis (ET) the 176 and 140 kDa fragments were increased in proportion to the intact 225 kDa subunit indicating increased proteolysis. Treatment of one ET patient with CCNU (Lomustine) decreased the platelet count and, to a lesser extent, the white blood cell count. This was associated with a correction of the bleeding time, a partial correction of the multimeric abnormality, and a lessening of vWF cleavage. In a patient with polycythemia rubra vera (PRV) the proportion of the 176 kDa fragment was increased to the upper limit of normal but there was no definite evidence of increased proteolysis. These studies provide evidence that proteolysis plays a role in the acquired von Willebrand's disease associated with the myeloproliferative syndrome. However, other mechanisms must also be considered.
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ACQUIRED von Willebrand's disease (vWD) has been described recently in patients with the myeloproliferative syndrome and high leukocyte and platelet counts.[1,2] The multimeric pattern of these patients' von Willebrand factor (vWF) resembled that of patients with inherited Type II vWD in that the largest and sometimes the intermediate multimers were missing.[1] As in normal plasma, each multimer could be resolved into three or more bands. The multimeric patterns of most patients showed a relative increase of the fastest moving band in each multimer, similar to IIA and IIB vWD. Normalization of the platelet and leukocyte count usually led to a progressive normalization of the multimeric pattern. In several patients, however, some abnormalities were still detectable when platelet counts were normal.

We have recently studied the proportion of intact vWF subunit, as compared to smaller proteolytic fragments in inherited Type II vWD variants.[3] Evidence for increased in vivo proteolysis, as compared to that of normal plasma vWF, was found in Types IIA and IIB. On the other hand, little or no proteolysis could be detected in Types IIC, IID, and IIE even though large multimers were also missing from plasma in these variants. In order to determine if proteolysis might contribute to the acquired absence of large vWF multimers seen in the myeloproliferative syndrome, we have examined the subunit composition of vWF from four patients in whom the large multimers were absent. Increased proteolysis was demonstrated in three individuals with essential thrombocytosis (ET), whereas no definite evidence of increased proteolysis could be detected in a fourth patient with polycythemia rubra vera (PRV). These results suggest not only that proteolysis participates in the pathogenesis of acquired vWD in association with the myeloproliferative disorder, but also that additional mechanisms should be considered.

MATERIALS AND METHODS

Reagents. Leupeptin was acquired from Chemicon (El Segundo, Calif); N-ethylmaleimide (NEM) from Sigma Chemical Co. (St. Louis, Mo); electrophoresis pure reagents from Bio-Rad (Richmond, Calif); rabbit antimouse IgG was from Zymed (San Francisco, Calif). All other reagents were of the highest grade available.

Patients. Patients studied included three women (Patients 1–3) with essential thrombocytosis (ET), aged 26, 45, and 69 years, and one woman (Patient 4) with polycythemia rubra vera (PRV), aged 69 years. Clinical laboratory data is presented in Table 1. Factor VIII, vWF:Ag, ristocetin cofactor activity (vWF:RCo), and bleeding times were determined as described.[1]

Preparation of plasma samples. Blood was drawn from a large antecubital vein. The blood was collected in polypropylene syringes with 1/10th final volume of 3.8% sodium citrate containing sufficient leupeptin, N-ethylmaleimide (NEM), and EDTA to give final concentrations in the blood of 1.0 mmol/L, 6 mmol/L, and 5 mmol/L, respectively. Platelet-poor plasma was obtained by immediate centrifugation at 3,200 rpm (approximately 2,700 g) for 20 minutes at 23 °C. The plasma was rapidly separated from the cells and stored at −70 °C. Platelet-rich plasma was prepared by immediate centrifugation at 2,200 rpm (approximately 1,100 g) for 75 seconds at 23 °C × 3, with aspiration and pooling of the platelet-rich plasma after each spin.

Immunoisolation of plasma vWF. vWF was immunoisolated by incubation of 1 to 5 mL of plasma with 1 to 2.5 mL of agarose beads covalently linked with anti-vWF monoclonal antibody, as described.[3] After extensive washing, the vWF was eluted with 2 mL of 2% SDS, 0.1 mol/L Tris (pH 8.0) at 60 °C, and the eluate was concentrated using Amicon Centricon 30 microconcentrators to a final volume of approximately 100 μL.

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Table 1. Clinical Data of the Four Patients With Myeloproliferative Disease Included in the Study

	1 (ET)			2 (ET)			
	4/10/85*	4/17/85	5/8/85	Pre DDAVP	30′ Post DDAVP	3 (ET)	4 (PRV)
Platelet count (× 10^3/mL)	2,240	1,960	348	2,760	2,745	1,765	545
WBC (× 10^3/mL)	19.2	21.5	16.9	13.2	13.4	14.4	16.2
RBC (× 10^{12}/mL)	5.64	5.53	5.49	4.10	3.88	5.02	6.13
Hct	37.7	36.7	36.4	38.9	36.6	38.8	38.9
Bleeding time (min)	>15	>15	8	10	5.5	10.5	>20
Factor VIII (U/dL)	92	110	176	74	225	114	50
vWF:Ag (U/dL)	112	84	171	52	215	62	36
vWF:RCo (U/dL)	64	42	166	54	138	64	56

Abbrev: essential thrombocytosis, ET; polycythemia rubra vera, PRV.
*CCNU (Lomustine) 160 mg given PO.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-5% polyacrylamide gels were prepared according to Laemmli.[4] The vWF was reduced by dithiothreitol (final concentration 65 mmol/L) for 15 minutes at 60 °C in pH 7.0 polyacrylamide gel sample buffer. Following SDS-5% polyacrylamide electrophoresis the samples were transferred to nitrocellulose and reacted with a pool of 55 anti-vWF monoclonal antibodies, all of which reacted with the reduced 225 kDa subunit.[3] The nitrocellulose was then reacted wtih ^{125}I-rabbit antimouse IgG antibody. The relative concentrations of the intact vWF subunit and its proteolytic fragments were estimated by identifying each band by autoradiography and then excising each band and counting them in a Packard Auto-Gamma Scintillation Spectrometer, United Technologies Instrument Co, Downer's Grove, IL. The plasma from each patient was processed on four separate occasions and the means and ranges of counts were determined from these combined analyses.

SDS-Agarose Electrophoresis for analysis of von Willebrand factor multimers was performed as described.[3]

RESULTS

Prior to therapy in all four patients, large vWF multimers were absent from plasma (Figs 1 and 2). In the three ET patients (patients 1, 2, and 3), the fastest moving band in each multimer was relatively increased. In the PRV patient (patient 4), the relative concentration of each band within a given multimer was similar to that of normal vWF.

In order to show that the presence or absence of cells in plasma did not affect the relative concentration of the vWF fragments, the following experiments were performed. vWF was immunopurified from plasma prepared by centrifugation of blood at 2,700 g for 15 minutes prior to freezing and from plasma that had been recentrifuged at 100,000 g prior to freezing. Comparison of the relative concentration of fragments showed no differences. Similarly, vWF was immunopurified from platelet-rich plasma that had been incubated for 18 hours at 37 °C and compared with vWF from plasma that had been doubly centrifuged as above and then similarly incubated. Again no differences were seen in the relative concentration of fragments.

Evaluation of reduced vWF immunopurified from patient plasmas showed that the relative proportions of the 176 and 140 kDa polypeptides were significantly increased in patients 1, 2, and 3, and that of the intact 225 kDa subunit decreased (Figs 3 and 4). In patient 4, the proportions of the subunit and fragments were within the normal range, although the 176 kDa fragment was at the upper limits of normal. No

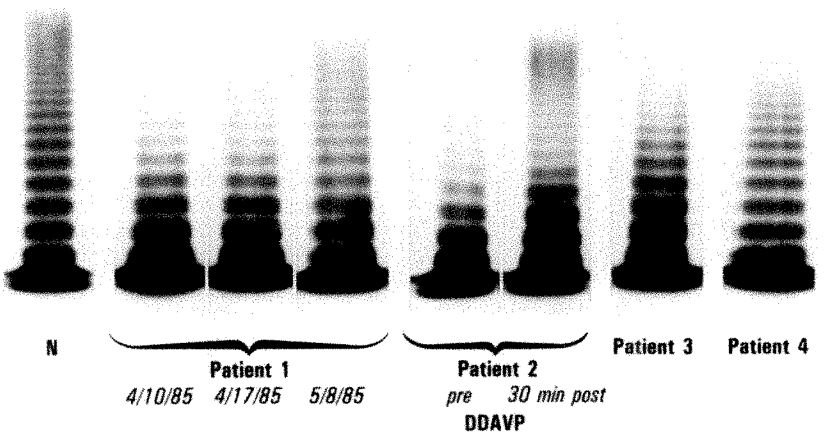

Fig 1. vWF multimeric analysis with 1.5% high gelling temperature agarose. The direction of electrophoresis is from top to bottom with the largest multimers at the top. It can be seen that the largest multimers present in normal plasma (N) are absent from patients 1, 2, 3, and 4. In patient 1 there was partial return of large and intermediate-sized multimers following treatment with CCNU (sample 5/8/85). Patient 2 showed transient return of large and intermediate-sized multimers post DDAVP.

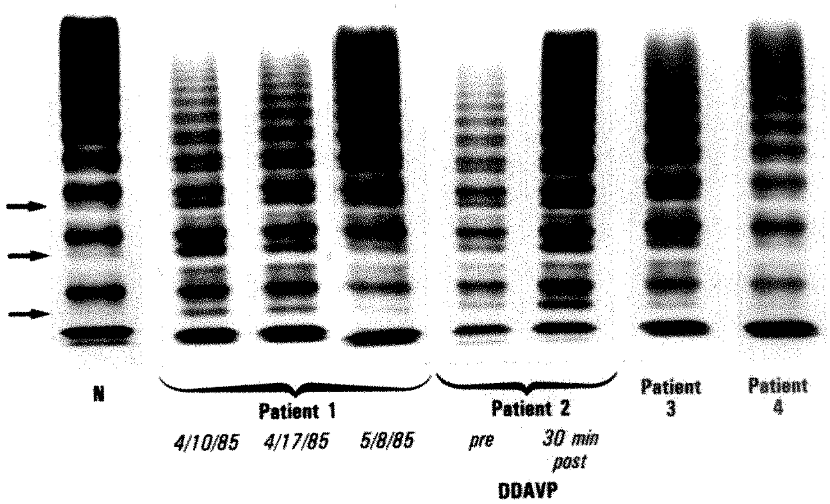

Fig 2. vWF multimeric analysis with 1.4% low gelling temperature agarose (LGT) agarose. The higher resolution obtained with LGT agarose separated each multimer into at least three bands. It can be seen in patients 1, 2, and 3 that the fastest moving band in each multimer (arrows) is relatively increased as compared to normal but that in patient 4 the pattern is similar to normal.

abnormality of the 189 kDa fragment was seen in any patient.

Patient 1 was treated with CCNU (Lomustine), following which the platelet count and bleeding time decreased to normal. The leukocyte count was also reduced but remained above normal (Table 1). These changes were accompanied by a correction of the bleeding time and partial normalization of the plasma vWF multimeric structure (Figs 1 and 2). Intermediate-sized multimers returned, although not in their usual quantity. The relative proportion of bands within each multimer also became more similar to that of normal vWF. These changes were associated with a partial return toward normal of the proportion of fragments in relation to the intact subunit (Figs 3 and 4).

1-desamino-8-D-arginine vasopressin (DDAVP) was administered to patient 2. This was followed by a shortening of the bleeding time from ten minutes to 5.5 minutes, a more than twofold increase in vWF:RCo, a threefold increase in Factor VIII, and a fourfold increase in vWF:Ag (Table 1). The largest multimers returned transiently. However, there was no correction of the relative proportions of the bands within each multimer nor was there a reduction in the proportion of vWF fragments in comparison with the intact subunit (Figs 3 and 4).

DISCUSSION

The participation of in vivo proteolysis in the pathogenesis of some forms of inherited vWF has been suggested by two recent studies. Gralnick and coworkers[6] provided evidence that Type IIA vWF was more susceptible to proteolysis than normal vWF. They, as well as Batlle and colleagues,[7] showed that in vitro loss of intermediate-sized multimers could be prevented in some patients by including NEM, leupeptin,

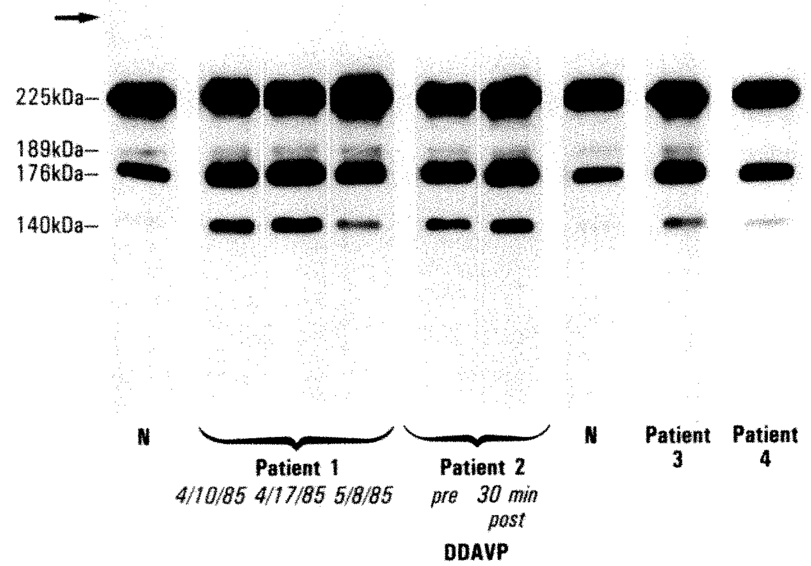

Fig 3. Reduced immunoisolated vWF following SDS-5% PAGE and immunoblotting. The 176 and 140 kDa bands were relatively increased in patients 1–3 as compared to the 225 kDa intact subunit. This visual impression was confirmed by γ-scintillation counting (Fig 4). There were no apparent significant increases of these bands in patient 4. No change in the 189 kDa band could be discerned in any patient.

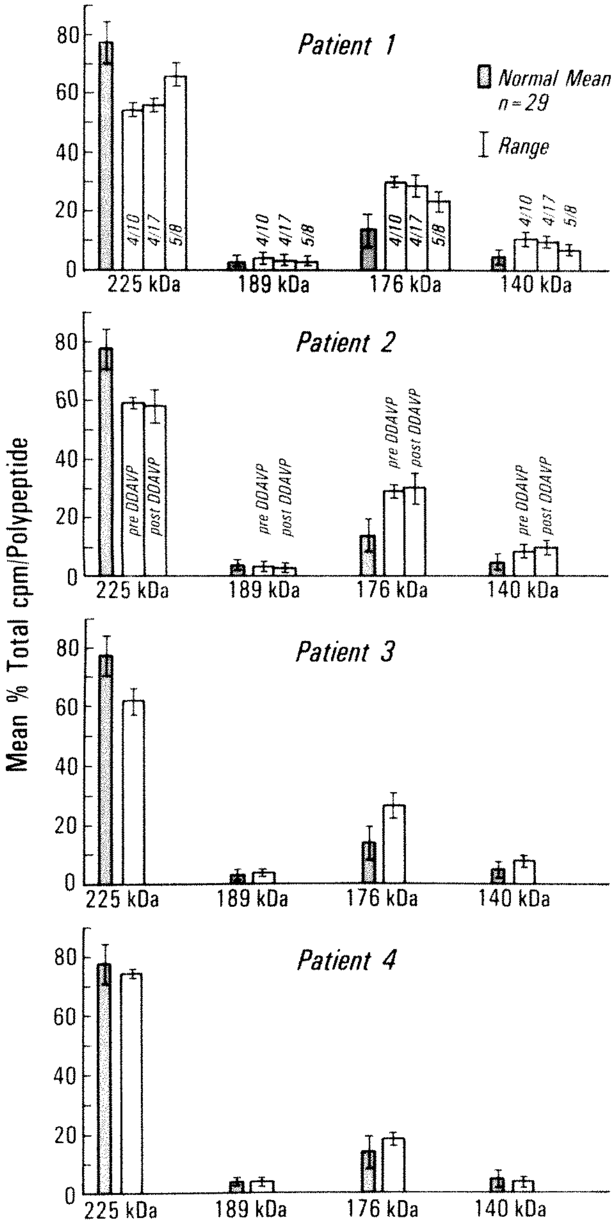

Fig 4. Relative proportion of counts in each of the bands shown in Fig 3 as determined by excising each band from nitrocellulose and measuring γ-scintillation counts. The means and ranges were from four separate immunoisolations, gels, and immunoblots for each patient. The normal mean was determined by performing the same analysis on samples from 29 different normal subjects. The 176 and 140 kDa bands were increased and the intact 225 kDa subunit decreased proportionately in patients 1–3. In patient 4 there was no increase, although the 176 kDa was at the upper limit of normal. No significant change was observed in the 189 kDa band in any patient.

and EDTA in the anticoagulant into which blood was collected. In unpublished studies, we have shown that in vitro generation of vWF fragments can also be suppressed by these inhibitors. Evidence for increased in vivo proteolysis of IIA and IIB vWF derived from studies of Zimmerman et al, who showed increased proportions of the 176 and 140 kDa fragments relative to the intact 225 kDa subunit in these two disorders.[3] On the other hand, little or no proteolysis of the 225 kDa subunit could be demonstrated in Types IIC, IID, and IIE vWD.[3]

Types IIA and IIB vWD have in common with the three essential thrombocytosis patients a similar alteration in the relative proportion of bands that comprise each multimer. In both the inherited vWD subtypes, as well as in the acquired form exhibited by these three patients, there was a relative increase in the fastest moving band in each multimer. This was associated in each case with evidence of increased proteolytic cleavage of vWF, as evidenced by increased proportions of the 176 and 140 kDa species. In contrast to Types IIA and IIB, there was no decrease in the relative quantity of the 189 kDa polypeptide.

Patient 4 differed from the others in that she had polycythemia rubra vera and did not show evidence of increased proteolysis of the vWF subunit, even though large multimers were absent from plasma. She also did not show the alteration in relative concentration of bands comprising each multimer. It is possible that the technique used for demonstrating increased proteolysis was not sufficiently sensitive to detect an abnormality in this case. Alternatively, the vWF abnormality in this patient may have a different basis from that of patients 1, 2, and 3.

The source and nature of the enzyme(s) responsible for vWF proteolysis in patients 1, 2, and 3 is unknown at present. Kunicki and coworkers have shown that the platelet calcium-activated protease can cleave vWF with resultant loss of large multimers.[8] Although each of the patients studied above had markedly elevated platelet counts initially, correction of the platelet count in patient 1 after treatment with CCNU was associated with only a partial diminution of the proteolytic and multimeric abnormalities. In addition, incubation of the vWF present in platelet-rich plasma produced no increase in proteolysis compared to acellular plasma. Moreover, the white blood count was also elevated in each of these patients and leukocytes are known to be the source of a number of enzymes with the potential of cleaving vWF.[9]

In summary, absence of large vWF multimers and alteration in the relative concentration of the bands comprising the multimers can be an acquired abnormality that may accompany the myeloproliferative syndrome. Proteolysis appears to contribute to this structural abnormality, although other causes may be operative.

REFERENCES

1. Budde U, Schaefer G, Mueller N, Egli H, Dent J, Ruggeri ZM, Zimmerman TS: Acquired von Willebrand's disease in the myeloproliferative syndrome. Blood 64:981, 1984

2. Leupin L, Beck, EA, Furlan M, Bucher U: Hamostasestorung mit verminderter Aktivitat des von Willebrand-Faktors bei myeloproliferativen Syndromen. Schweiz Med Wochenschr 113:713, 1983

3. Zimmerman TS, Dent JA, Ruggeri ZM, Nannini LH: Subunit composition of plasma von Willebrand factor. Cleavage is present in normal individuals, increased in IIA and IIB von Willebrand

disease, but minimal in variants with aberrant structure of individual oligomers (Types IIC, IID and IIE). J Clin Invest 77:947, 1986

4. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

5. Ruggeri ZM, Zimmerman TS: The complex multimeric composition of Factor VIII/von Willebrand factor. Blood 57:1140, 1981

6. Gralnick HR, Williams SB, McKeown LP, Maisonneuve P, Jenneau C, Sultan Y, Rick ME: In vitro correction of the abnormal multimeric structure of vWf in Type IIA vWd. Proc Natl Acad Sci USA 82:5968, 1985

7. Batlle J, Lopez-Fernandez MF, Campos M, Justica B, Borges C, Navarro JL, Diaz Cremades JM, Kasper CK, Dent JA, Ruggeri ZM, Zimmerman TS: The heterogeneity of Type IIA von Willebrand disease: Studies with protease inhibitors. Blood (this issue)

8. Kunicki TJ, Montgomery RR, Shulleck J: Cleavage of human von Willebrand factor by platelet calcium-activated protease. Blood 65:352, 1985

9. Thompson EA, Howard MA: Proteolytic cleavage of human von Willebrand factor induced by enzyme(s) released from polymorphonuclear cells. Blood 67:1281, 1986

Mechanism of Protein C–Dependent Clot Lysis: Role of Plasminogen Activator Inhibitor

By Yoichi Sakata, David J. Loskutoff, Candece L. Gladson, Carla M. Hekman, and John H. Griffin

The mechanism by which activated protein C stimulates fibrinolysis was studied in a simple radiolabeled clot lysis assay system containing purified tissue-type plasminogen activator, bovine endothelial plasminogen activator inhibitor (PAI), plasminogen, ^{125}I-fibrinogen and thrombin. Fibrinolysis was greatly enhanced by the addition of purified bovine activated protein C; however, in the absence of PAI, activated protein C did not stimulate clot lysis, thus implicating this inhibitor in the mechanism. In clot lysis assay systems containing washed human platelets as a source of PAI, bovine-activated protein C–dependent fibrinolysis was associated with a marked decrease in PAI activity as detected using reverse fibrin autography. Bovine-activated protein C also decreased PAI activity of whole blood and of serum. In contrast to the bovine molecule, human-activated protein C was much less profibrinolytic in these clot lysis assay systems and much less potent in causing the neutralization of PAI. This species specificity of activated protein C in clot lysis assays reflect the known in vivo profibrinolytic species specificity. When purified bovine-activated protein C was mixed with purified PAI, complex formation was demonstrated using immunoblotting techniques after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. These observations suggest that a major mechanism for bovine protein C–dependent fibrinolysis in in vitro clot lysis assays involves a direct neutralization of PAI by activated protein C.

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PROTEIN C (PC) is a plasma protease zymogen that can exhibit both anticoagulant and profibrinolytic activities.[1-6] Since an inherited deficiency of PC is associated with venous thromboembolic disease,[7,8] PC functions physiologically as a major antithrombotic factor. This antithrombotic activity may derive from either its anticoagulant action or its profibrinolytic activity, or from both properties. The mechanism of action of activated PC (APC) as an anticoagulant in plasma involves the proteolytic inactivation of Factors V and VIII.[4,9,10] Less clear is the mechanism of action of APC in stimulating fibrinolysis. Infusion of bovine APC into dogs elevated circulating lysine-adsorbable plasminogen activator (PA).[6] Comp and Esmon suggested that APC interacts with whole blood to generate a messenger that stimulates the release of PA from the endothelium.[6] APC added to blood in vitro enhanced clot lysis assays by unspecified mechanisms that were suggested to require the participation of blood cells.[11,12] Our laboratories have been studying the functional activities of PC[4,13] and of components of the fibrinolytic system.[14,15] Since fibrinolytic activity measurements are based on the balance between PAs and plasminogen activator inhibitors (PAIs), enhanced fibrinolysis may result from PAI decreases as well as PA increases.[15] Bovine endothelial cells,[16] human platelets,[17] and human serum[17] contain a potent PAI and the bovine endothelial PAI has been purified.[18] The present study was undertaken to determine the potential influence of APC on PAI in clot lysis assays. The results demonstrate that bovine APC neutralizes PAI activity, and in the process, stable APC-PAI complexes are formed. These interactions may explain the profibrinolytic activity of APC in several types of blood clot lysis assays.

MATERIALS AND METHODS

Reagents. All chemicals were the best analytic grade commercially available. Tissue culture materials were purchased from the following sources: Plasticware from Corning (Corning, NY); media from Flow Laboratories (McLean, VA); calf serum, trypsin, penicillin, and streptomycin from GIBCO (Grand Island, NY). Materials were obtained as follows: Proplex^{R} from Hyland Therapeutics; hirudin (grade IV), bovine serum albumin (essentially fatty acid free), Triton X-100, Concanvalin A-Sepharose, and chloramine-T from Sigma (St. Louis, MO); Bio-Rex 70 from Bio-Rad Laboratories (Richmond, Calif); LPG-agarose from Miles Laboratories (Naperville, IL); Blue-Sepharose CL-6B, Sepharose CL-2B, and DEAE Sephadex A50 from Pharmacia Fine Chemicals (Piscataway, NJ); and S2366 (pyro-Glu-Pro-Arg-paranitroanilide) from Kabi (Stockholm).

Preparation of whole blood and serum. Blood was collected from antecubital veins of normal subjects into polypropylene tubes containing 0.1 vol of 3.8% sodium citrate. Serum was obtained by adding thrombin (final concentration 0.5 U/mL) and $CaCl_2$ (final concentration 15 mmol/L) to blood for ten minutes at 37 °C. After ten minutes, hirudin (final concentration 100 U/mL) was added to the mixture and clots were removed by centrifugation at 2000 g for 20 minutes.

Preparation of gel-filtered platelets and platelet releasates. Blood was obtained from normal subjects and mixed with 1/6 volume acid citrate dextrose. Citrated blood was centrifuged at 160 × g to obtain platelet-rich plasma that was collected and centrifuged at 660 × g. The platelet pellet was carefully resuspended in calcium-free HEPES-Tyrode's buffer, pH 6.5, and then passed over a Sepharose CL-2B column that had been equilibrated with calcium-free HEPES-Tyrode's buffer, pH 7.3.[19] Platelets were counted with a Coulter counter (Coulter Electronics, Inc, Hialeah, Fla). To prepare platelet releasate, gel-filtered platelets were diluted with 0.1 mol/L Tris HCl, pH 8, containing 0.01% Tween 80 to a final concentration of 5×10^8 platelets/mL, and then the platelets were incubated with thrombin (final concentration 0.5 U/mL) for ten minutes at 37 °C. Then hirudin was added (final concentration 10 U/mL) and the samples were centrifuged at 1,800 × g for 30 minutes at 4 °C to remove the platelets and obtain platelet releasates.

Proteins. Bovine PC was purified from bovine plasma by using barium citrate adsorption, ammonium sulfate precipitation, DEAE-Sephadex,[2] and Blue-Sepharose CL-6B chromatography. Human PC was purified from Proplex^{R} as described elsewhere.[10] The PC and APC appeared >95% homogeneous on 9% polyacrylamide gels in the presence of sodium dodecyl sulfate.

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APC was prepared by activation of PC with α-thrombin at an enzyme-to-substrate weight ratio of 1:5. Activation was monitored as the appearance of amidolytic activity toward the chromogenic substrate S2366. Activity was measured using a Cary 210 Spectrophotometer as the initial ΔA_{405}/min at 37 °C. Thrombin was removed from APC by chromatography on a column of Bio-Rex 70. Human APC (hAPC) was also tested for its anticoagulant activity toward human plasma in an activated partial thromboplastin time assay. Plasminogen was purified by affinity chromatography on lysine-Sepharose.[20] Fibrinogen, human fraction I-4 prepared according to the method of Blomback and Blomback,[21] was used as the fibrinogen after removing contaminating plasminogen using lysine-Sepharose. Human urokinase (World Health Organization 1st International Reference Preparation) was purchased from the National Institute for Biological Standards and Control, London.

PAI was purified from 24-hour CM collected from bovine aortic endothelial cells (BAEs)[22] using Concanavalin A-Sepharose and preparative SDS-polyacrylamide gel electrophoresis (PAGE) as described elsewhere.[18] PAI used for immunoblotting studies was purified without exposure to SDS and was exposed to guanidine HCl to generate active PAI (Hekman and Loskutoff, unpublished). Human t-PA was purified from cultured human melanoma cells grown in the presence of Trasylol (Calbiochem-Behring, La Jolla, CA) according to the method of Rijken and Collen.[23] It had a specific activity of ~100,000 U/mg of protein, determined by using the urokinase reference standard, and consisted primarily of the single chain molecule.

Radioiodination. Purified fibrinogen and goat antirabbit IgG were radioiodinated using the chloramine-T method.[24] The labelled fibrinogen preparation had a specific activity of ~182 μCi/mg and was greater than 94% clottable.

Measurement of plasmin-induced clot lysis. 3.7 mL of fibrinogen (fraction I-4, 10 mg/mL) was mixed with 120 μg of radiolabeled fibrinogen. 250 μL of this mixture was mixed with 20 μL of a solution containing plasminogen (10 mg/mL) and hAPC or bovine APC (bAPC) (final concentration 3.4 μg/mL). Various amounts of t-PA and various amounts of purified PAI and/or gel-filtered platelets (final concentration 5×10^8/mL) were added to this mixture. Final volume was adjusted by adding 0.1 mol/L Tris-HCl, pH 8.1, containing Tween 80 (final concentration 0.01%) and bovine albumin (final concentration 1 mg/mL) to 1 mL. The mixture was clotted with α-thrombin (final concentration 0.5 U/mL) and $CaCl_2$ (final concentration 2.5 mmol/L. The mixture was allowed to clot at 37 °C for ten minutes, and then the clot was freed from the wall of the tube by rimming the clot with a bamboo stick. To measure fibrinolysis, 50 μL aliquots of serum were removed from around the clot at intervals for counting the soluble radioactivity. Results were expressed as the percent release of total radioactivity, which was calculated from the observed radioactivity, applying a correction for the influence of repeated subsampling upon the volume of the supernatant. Visible clot lysis was also monitored.

SDS-PAGE. SDS-PAGE on slab gels was carried out using resolving gels of 9% acrylamide and stacking gels of 4% acrylamide according to Laemmli.[25]

Reverse fibrin autography. Fibrin-agar indicator gels were prepared and reverse fibrin autography assays were performed as previously described.[26-28] The opaque white lysis-resistant zones in the indicator gel result from the presence of inhibitors in the SDS polyacrylamide slab gel.[26]

Western blotting. Reaction mixtures of purified bovine APC and bovine PAI were analyzed by Western blotting using rabbit antiserum against purified APC and PAI. 10 μL of 260 nmol/L PAI in PBS was added to 5 μL of 280 nmol/L APC in 0.05 mol/L Tris, 0.1 mol/L NaCl, 0.1% Triton X-100, 0.1% BSA, pH 7.4, and then incubated at 37 °C for 20 minutes. As controls, identical samples of PAI and APC alone were incubated for 20 minutes at 37 °C. After fractionation by SDS-PAGE, proteins in the gels were electrophoretically transferred (50 v, 1.5 hours) to nitrocellulose essentially as described[29] using an electroblot apparatus (Hoffer Scientific Instruments, San Francisco). The transfer buffer consisted of 50 mmol/L Tris-HCl, 95 mmol/L glycine, 20% methanol, and 0.1% SDS, pH 8.3. At the end of the transfer period, the nitrocellulose sheets were blocked by incubating them in 5% (w/v) nonfat dry milk solution containing 0.01 mol/L sodium phosphate, pH 7.4, 0.15 mol/L NaCl, 1 μmol/L thimerosal, 1 μmol/L para-amidinophenylmethylsulfonylfloride, 0.02% NaN_3 (blocking buffer). The nitrocellulose membranes were then incubated for four hours at 22 °C in rabbit antibovine PAI serum diluted 1:500 or rabbit antibovine PC antiserum diluted 1:4000. After washing three times with blocking buffer, the sheets were incubated for 1.5 hours with ^{125}I-labeled goat antirabbit IgG (Cappel Laboratories, Cochranville, PA), washed extensively, and subjected to autoradiography using Kodak X-Omat film.

RESULTS

The ability of purified bovine APC to stimulate fibrinolysis in a simple clot lysis assay system composed of purified t-PA, PAI, ^{125}I-fibrinogen, plasminogen, and thrombin was studied. The profibrinolytic activity of APC in the presence of PAI was easily seen during the time course of clot lysis of such mixtures (Fig 1). For example, the presence of PAI at 10 ng/mL retarded clot lysis in the absence of APC. However, in the presence of bovine APC, this inhibition of clot

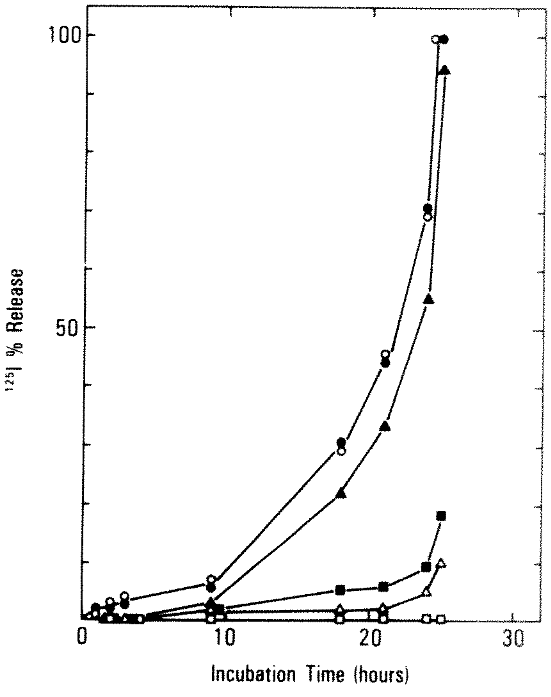

Fig 1. Effect of bovine APC on clot lysis assays containing purified t-PA, PAI, and APC. Purified PAI, whenever indicated, was added to a mixture containing ^{125}I-fibrinogen and plasminogen. t-PA (0.05 ng/mL) was added to the mixture simultaneously with or without 3.4 μg/mL APC. Immediately after adding t-PA and APC, samples were clotted with thrombin and $CaCl_2$. Clot lysis is indicated as ^{125}I % release. PAI was present at 0 (○, ●), 10 ng/mL (△, ▲), or 20 ng/mL (□, ■). The presence of APC is indicated by solid symbols, the absence by open symbols.

lysis by PAI was greatly reduced, suggesting that APC neutralized the effect of PAI. Consistent with this suggestion was the observation that APC was not profibrinolytic in the absence of PAI (Fig 1, open v solid circles). In control studies (data not shown), bovine APC alone had no effect on the functional activity of t-PA, plasminogen, or ^{125}I-fibrinogen, or on the apparent M_r of these molecules during SDS-PAGE. Hence, based on clot lysis assays composed of only purified proteins, the data in Fig 1 suggest that bovine APC directly neutralized the bovine endothelial PAI.

Human platelets contain a PAI that is immunologically related to the purified bovine PAI and that is released from platelets by thrombin.[17] The ability of purified bovine and human APC to promote fibrinolysis and to neutralize platelet PAI in clots containing washed human platelets, t-PA, ^{125}I-fibrinogen, thrombin, and APC was studied (Fig 2). Bovine APC enhanced clot lysis in such mixtures as measured by the release of soluble ^{125}I-fibrin degradation products or visualized as clot disappearance (Fig 2). The profibrinolytic activity of human APC in these clot lysis assays was weak compared to bovine APC but reproducible. Human APC caused fragmentation of the clot under conditions where the control remained a single solid clot, but the rate of release of soluble ^{125}I-fragments was not enhanced in this experiment. Thus, bovine APC appeared much more potent than human APC under these assay conditions.

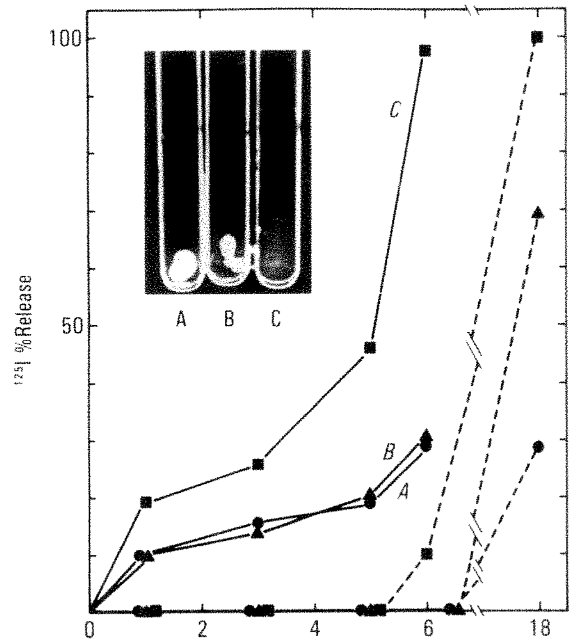

Fig 2. Effect of APC on clot lysis assays containing washed human platelets. Gel-filtered platelets (final concentration 5 × 10^8 platelets/mL) were added to a reaction mixture containing ^{125}I-fibrinogen and plasminogen. Then 0.2 ng/mL of t-PA (dashed line) or 1 ng/mL of t-PA (solid lines) was added as well as no APC (●), 3.4 µg/mL of human APC (▲), or 3.4 µg/mL of bovine APC (■). Then samples were immediately clotted with thrombin and $CaCl_2$. Clot lysis is reported as ^{125}I % release. Inset, photos of reaction mixtures in test tubes after six hours incubation of the samples containing 1 ng/mL t-PA are shown for no APC (A), human APC (B), or bovine APC (C).

The ability of human and bovine APC to neutralize PAI activity released from human platelets was determined. Changes in PAI were monitored using reverse fibrin autography,[26] a technique that allows direct visualization of PAI activity (Fig 3). When bovine APC was added to platelet releasate, PAI activity was decreased in a dose-dependent manner to levels that were less than 20% of untreated controls. The effect of human APC was less pronounced than that of bovine APC but it was significantly and reproducibly different from controls. The ability of APC to reduce the PAI activity of human whole blood and serum was also studied using reverse fibrin autography (Fig 4). Bovine APC abolished the detectable PAI activity of whole blood or serum (Fig 4), whereas human APC at similar concentrations had a rather weak effect under the conditions employed (Fig 4 and data not shown). In control studies, it was verified that the human APC used in these studies was fully active in anticoagulant and amidolytic assays. Thus, bovine APC was much more potent than human APC in the neutralization of human platelet PAI.

Since aforementioned results demonstrate that APC inactivates PAI, immunoblotting experiments were designed to see if inactivation of PAI by APC was associated with the formation of stable APC-PAI complexes. When purified PAI and purified bovine APC were mixed together and subjected to immunoblotting analyses, two new bands at approximately 93,000 and 100,000 apparent Mr were seen on immunoblots using anti-PAI antibodies (Fig 5, lane 3) and anti-PC antibodies (Fig 5, lane 4). These Mr values

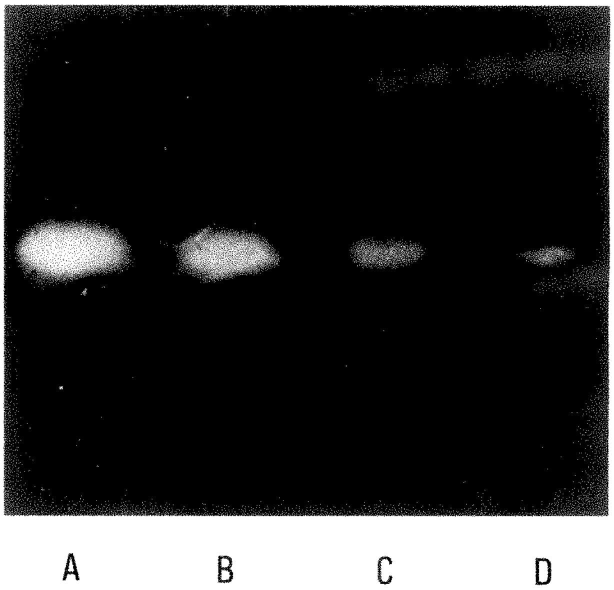

Fig 3. Effect of APC on the PAI activity derived from washed stimulated human platelets. Human or bovine APC was added to platelet releasate obtained as described in the Methods, and the mixtures were incubated for 30 mintues at 37 °C. After this incubation, 50 µL of the samples were subjected to SDS-PAGE and analyzed using reverse fibrin autography to visualize PAI activity. Lane A, control, platelet releasate without APC; Lane B, platelet releasate plus human APC (3.4 µg/mL); Lane C, platelet releasate plus bovine APC (0.85 µg/mL); Lane D, platelet releasate plus bovine APC (3.4 µg/mL).

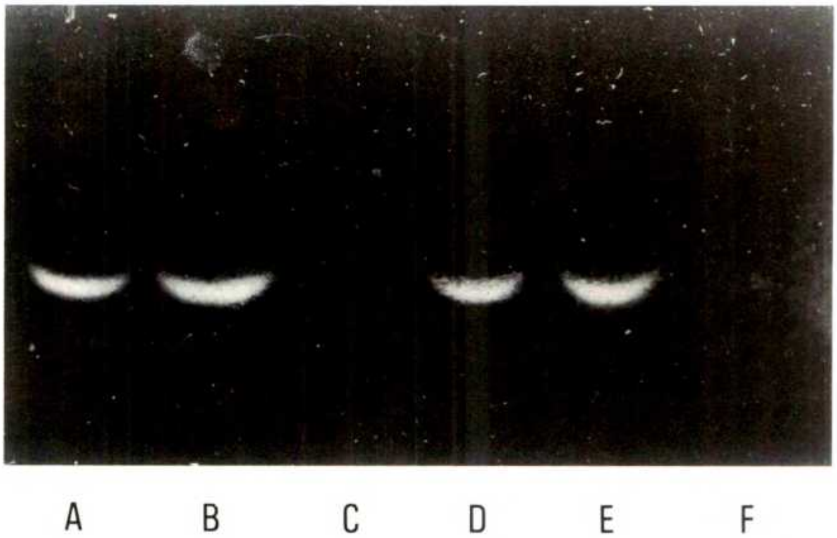

Fig 4. Effect of APC on the PAI activity of whole blood and of serum derived from whole blood. Human or bovine APC (final concentration 3.4 $\mu g/mL$) was added to citrated whole blood and the mixture was clotted with thrombin (final concentration 0.5 U/mL) and $CaCl_2$ (final concentration 15 mmol/L) and then incubated at 37 °C for 15 minutes. Then hirudin (final concentration 100 U/mL) was added to the mixture and the samples were centrifuged at 1900 × g for 15 minutes at 4 °C to obtain serum. 35 μL of these samples were subjected to SDS-PAGE and analyzed using reverse fibrin autography to visualize PAI activity. Similarly, human or bovine APC (final concentration 3.4 $\mu g/mL$) was added to serum that had been made from citrated whole blood as described in Methods. Samples were incubated for 15 minutes at 37 °C and 35 μL of these samples were fractionated by SDS-PAGE and analyzed using reverse fibrin autography. Lane A, Whole blood control without added APC; Lane B, whole blood plus human APC; Lane C, whole blood plus bovine APC; Lane D, serum control without added APC; Lane E, serum plus human APC; Lane F, serum plus bovine APC.

correspond approximately to the addition of monomeric PAI (~44,000 apparent Mr in lane 1, Fig 5) and APC (~53,000 apparent Mr in Fig 5). Control studies showed that the band at 83,000 apparent Mr in lane 2, Fig 5, seen for PAI alone and incubated 20 minutes is due to a time-dependent dimerization of PAI since PAI exhibited one band (lane 1, Fig 5) without incubation. The band at 93,000 apparent Mr was detected equally well by anti-PAI and anti-PC antibodies, whereas the band at 100,000 apparent Mr was more sensitive to anti-PC antibodies than to anti-PAI antibodies. The comigration of PAI and APC antigenic determinants at 93,000 and 100,000 Mr appears to represent complexes of PAI and APC. The presence of these two different PAI-APC complexes may represent the presence of several forms of APC (lane 5, Fig 5).

DISCUSSION

The addition of APC to clot lysis assay mixtures containing whole blood or platelet-rich plasma was shown previously to enhance clot lysis.[11,12] The results presented in this report lead to the hypothesis that bovine APC stimulates in vitro clot lysis in these systems by neutralizing the PAI present in clots containing whole blood, serum, platelet-rich plasma, or washed platelets. This hypothesis is based on the findings

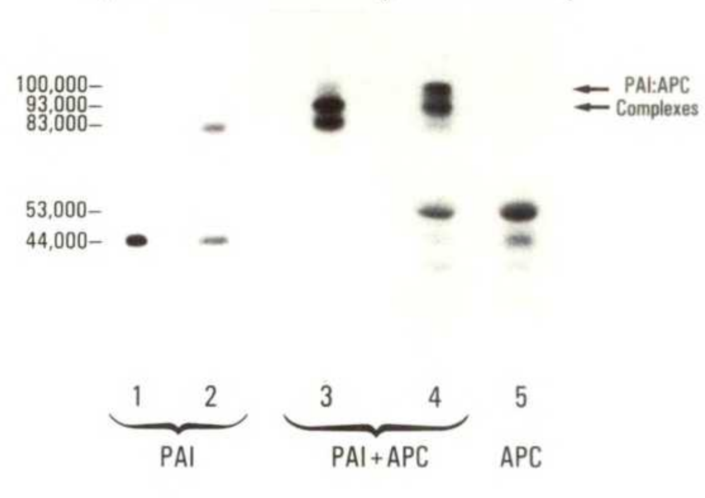

Fig 5. Immunoblotting studies of APC-PAI complexes. Lanes 1, 2, and 3 were developed with anti-PAI antiserum and lanes 4 and 5 were developed with anti-PC. Lanes 1 and 2 contained PAI alone incubated for 0 minutes (lane 1) or 20 minutes (lane 2) at 37 °C. Lanes 3 and 4 each contained a mixture of PAI and APC that had been allowed to react for 20 minutes at 37 °C. Lane 5 contained APC alone incubated for 20 minutes at 37 °C.

that APC is not profibrinolytic in the absence of PAI (Fig 1) and that PAI activity is decreased in a dose-dependent manner by bovine APC in clot lysis reaction mixtures. For example, bovine APC enhances clot lysis in the presence of platelets (Fig 2), a potent source of PAI.[17] This PAI is released from platelets by physiological concentrations of thrombin and is immunologically related to the bovine endothelial PAI.[17] Moreover, the addition of bovine APC to other samples containing PAI, such as to blood serum (Fig 4), or to conditioned media or extracts derived from endothelial cells,[27,28,30] results in a rapid, dose-dependent loss of PAI activity as revealed by reverse fibrin autography. Since complexes between PAI and PA, once formed, are resistant to dissociation by APC (unpublished data), it appears that APC acts only to prevent the initial interaction of PAI with PA. Taken together, the available observations strongly suggest that APC neutralizes PAI and, in so doing, allows t-PA to activate plasminogen and initiate clot lysis.

Since APC neutralizes PAI and since the former molecule is a protease and the latter is a protease inhibitor, we predicted that the two molecules might form an enzyme-inhibitor complex. Studies using immunoblotting techniques and purified bovine APC and PAI directly demonstrate the formation of bovine APC-PAI complexes of approximately 93,000 and 100,000 apparent Mr (Fig 5). Further studies will be needed to describe in detail the molecular mechanisms involved in complex formation.

The species specificity of the anticoagulant activity of APC is quite remarkable,[3,4,9] and as seen here the profibrinolytic activity of human APC is much less than that of bovine APC in clot lysis assays. In this regard, human APC infused into spider monkeys does not elevate PA levels,[32] whereas elevation of fibrinolytic activity is observed when bovine APC is infused into dogs.[5,6,33] Possible decreases of PAI during in vivo infusion of APC have been studied or reported,[5,6,32,33] so it is not yet clear what effect bovine or human APC may exert in vivo in different species on the levels of PAIs. In addition, it is not known how efficiently human or bovine APC is neutralized in vitro or in vivo by the bovine, human, or monkey plasma inhibitor of APC described by Suzuki et al[34] for human plasma.

The profibrinolytic activity of bovine APC has been described in three different assays systems: in vivo canine infusions of APC,[5,6] in vitro blood and plasma clot lysis mixtures containing APC,[11,12,35] and the addition of APC to cultured bovine endothelial cells or to conditioned media from such cells.[27,28,30] A quantitative comparison of the profibrinolytic activity of bovine APC in the three positive assays systems has not been made, but it was suggested that the activity observed for in vivo canine infusions could not be accounted for by what Comp and Esmon considered the weaker activity seen when bovine APC was added in vitro to canine blood.[6,33] The in vivo infusion studies of bovine APC showed an apparent increase of lysine-adsorbable PA activity[6] and the cultured endothelial cell studies showed both an apparent increase of each of the different observable forms of PA on fibrin autography gels and a decrease of PAI.[27,28,30] The in vitro clot lysis assays described here using blood, platelet-rich plasma, washed platelets, or only purified assay components here showed a decrease of PAI (Figs 3 and 4). Thus, we currently hypothesize that the major mechanism of profibrinolytic activity of bovine APC involves the direct neutralization of PAI in in vitro blood clot lysis assays. A similar direct effect has also been demonstrated in cultured endothelial cells.[28,30] The profibrinolytic activity of APC observed with in vivo canine infusions may involve either the direct neutralization of PAI by APC or an increase in the release of tissue PA from endothelial cells, or both of these effects.

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REFERENCES

1. Mammen EF, Thomas WR, Seegers WH: Activation of purified prothrombin to autoprothrombin I or autoprothrombin II (platelet cofactor II or autoprothrombin II-A). Thromb Diath Haemorrh 5:218, 1960

2. Stenflo J: A new vitamin K-dependent protein: Purification from bovine plasma and preliminary characterization. J Biol Chem 251:355, 1976

3. Kisiel W, Canfield WM, Ericsson LH, Davie EW: Anticoagulant properties of bovine plasma protein C following activation by thrombin. Biochemistry 16:5824, 1977

4. Marlar RA, Kleiss AJ, Griffin JH: Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. Blood 59:1067, 1982

5. Seegers WH, McCoy LE, Groben HD, Sakuragawa N, Agrawal BBL: Purification and some properties of autoprothrombin II-A: An anticoagulant perhaps also related to fibrinolysis. Thromb Res 1:443, 1972

6. Comp PC, Esmon CT: Generation of fibrinolytic activity by infusion of activated protein C into dogs. J Clin Invest 68:1221, 1981

7. Griffin JH, Evatt B, Zimmerman TS, Kleiss AJ, Wideman C: Deficiency of protein C in congenital thrombotic disease. J Clin Invest 68:1370, 1981

8. Broekmans AW, Veltkamp JJ, Bertina R: Congenital protein C deficiency and venous thromboembolism. N Engl J Med 309:340, 1983

9. Stenflo J: Structure and function of protein C. Semin Thromb Hemostasis 10:109, 1984

10. Fulcher CA, Gardiner JE, Griffin JH, Zimmerman TS: Proteolytic inactivation of human Factor VIII procoagulant protein by activated human protein C and its analogy with Factor V. Blood 63:486, 1984

11. Taylor FB, Esmon CT, Lockhart ML: Optimum conditions for lysis in whole blood clots, in Mann KG, Taylor FB (eds): Regulation of Coagulation (vol 8). New York, Elsevier North Holland, 1980, pp 573–581

12. Taylor FB, Carroll RC, Gerrard J, Esmon CT, Radcliffe RD: Lysis of clots prepared from whole blood and plasma. Fed Proc 40:2092, 1981

13. Gardiner JE, Griffin JH: Human protein C and thromboem-

bolic disease, in Brown E (ed): Progress in Hematology (vol XIII). Orlando, Fla, Grune & Stratton, 1983, pp 265–278

14. Loskutoff DJ, Levin EG, Mussoni L: Fibrinolytic components of cultured endothelial cells, in Nossel HL, Vogel H (eds): Pathobiology of the Endothelial Cell. Orlando, Fla, Academic Press, 1982, pp 167–182

15. Loskutoff DJ: The fibrinolytic system of cultured endothelial cells: Deciphering the balance between plasminogen activation and inhibition, in Prog. in Fibrinolysis (vol 7). London, Churchill-Livingstone, 1985, pp 15–22

16. Loskutoff DJ, van Mourik JA, Erickson LA, Lawrence DA: Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. Proc Natl Acad Sci USA 80:2956, 1983

17. Erickson LA, Ginsberg MH, Loskutoff DJ: Detection and partial characterization of an inhibitor of plasminogen activator in human platelets. J Clin Invest 74:1465, 1984

18. van Mourik JA, Lawrence DA, Loskutoff DJ: Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J Biol Chem 259:14914, 1984

19. Greengard JS, Griffin JH: Receptors for high molecular weight kininogen on stimulated washed human platelets. Biochemistry 23:6863, 1984

20. Brockway WJ, Castellino FJ: Measurement of the binding of antifibrinolytic amino acids to various plasminogens. Arch Biochem Biophys 151:194, 1972

21. Blomback B, Vestermark A: Isolation of fibrino-peptides by chromatography. Arkiv Kemi 12:173, 1958

22. Loskutoff DJ: Effect of thrombin on the fibrinolytic activity of cultured bovine endothelial cells. J Clin Invest 64:329, 1979

23. Rijken DC, Collen D: Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem 256:7035, 1981

24. Greenwood FC, Hunter WM, Glover JS: The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J 89:114, 1963

25. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 277:680, 1970

26. Erickson LA, Lawrence DA, Loskutoff DJ: Reverse fibrin autography: A method to detect and partially characterize protease inhibitors after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 137:454, 1984

27. Sakata Y, Curriden SA, Lawrence DA, Loskutoff DJ, Griffin JH: Protein C–dependent fibrinolytic activity of cultured bovine and human endothelial cells. Circulation II:205, 1984 (abstr 818)

28. Sakata Y, Curriden SA, Lawrence DA, Griffin JH, Loskutoff DJ: Activated protein C stimulates the fibrinolytic activity of cultured endothelial cells and decreases antiactivator activity. Proc Natl Acad Sci USA 82:1121, 1985

29. Towbin H, Gordon J: Immunoblotting and dot immunobinding—current status and outlook. J Immunol Methods 72:313, 1984

30. van Hinsbergh VWM, Bertina RM, van Wijngaarden A, van Tilburg JH, Emeis JJ, Haverkate F: Activated protein C decreases plasminogen activator-inhibitor activity in endothelial cell-conditioned medium. Blood 65:444, 1985

31. Hekman CM, Loskutoff DJ: Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. J Biol Chem 260:11581, 1985

32. Colucci M, Sassen JM, Collen D: Influence of protein C activation on blood coagulation and fibrinolysis in squirrel monkeys. J Clin Invest 74:200, 1984

33. Comp PC: Animal studies of protein C physiology. Semin Thrombos Hemostasis 10:149, 1984

34. Suzuki K, Nishioka J, Hashimoto S: Protein C inhibitor: Purification from human plasma and characterization. J Biol Chem 258:163, 1983

35. Taylor FB, Lockhart MS: A new function for activated protein C: Activated protein C prevents inhibition of plasminogen activators by releasate from mononuclear leukocytes—platelet suspensions stimulated by phorbol diester. Thromb Res 37:155, 1985

Expression of Fibrinogen Receptors During Activation and Subsequent Desensitization of Human Platelets by Epinephrine

By Sanford J. Shattil, Harvey J. Motulsky, Paul A. Insel, Lawrence Flaherty, and Lawrence F. Brass

Epinephrine causes platelet aggregation and secretion by interacting with α_2-adrenergic receptors on the platelet surface. Platelet aggregation requires the binding of fibrinogen to a specific receptor on the membrane glycoprotein IIb-IIIa complex. Although the IIb-IIIa complex is identifiable on the surface of resting platelets, the fibrinogen receptor is expressed only after platelet activation. The current studies were designed to examine the effect of occupancy of platelet α_2-adrenergic receptors by epinephrine on the expression of fibrinogen receptors and on the aggregation of platelets. The ability of epinephrine to induce the expression of fibrinogen receptors was studied under two different conditions: acute stimulation (<1 min) and prolonged stimulation (50 to 90 min), the latter of which is associated with a reduction or "desensitization" of the platelet aggregation response. Expression of the fibrinogen receptor was monitored with ^{125}I-fibrinogen as well as with ^{125}I-PAC-1 (PAC-1), a monoclonal antibody that binds to the glycoprotein IIb-IIIa complex only after platelets are activated. Epinephrine caused an immediate increase in PAC-1 and fibrinogen binding that was dependent on occupancy of the α_2-receptor by epinephrine and on the presence of extracellular free Ca (K_{Ca} = 30 μmol/L). By itself, 1 mmol/L Mg was unable to support induction of the fibrinogen receptor by epinephrine. However, it did decrease the Ca requirement by about two orders of magnitude. Prolonged stimulation of unstirred platelets by epinephrine led to a 70% decrease in the aggregation response when the platelets were subsequently stirred. Despite their decreased aggregation response, desensitized platelets bound PAC-1 and fibrinogen normally, indicating that the loss of aggregation was not due simply to a decrease in fibrinogen receptor expression. Although desensitization was not affected by pretreatment of the platelets with aspirin, it was partially prevented when extracellular Ca was chelated by EDTA during the long incubation with epinephrine. These studies demonstrate that once platelet α_2-adrenergic receptors are occupied by epinephrine, extracellular Ca is involved in initiating the aggregation response by supporting the induction of the fibrinogen receptor and the binding of fibrinogen. Furthermore, Ca-dependent reactions subsequent to fibrinogen binding may be necessary for maximal platelet aggregation and are impaired when platelets become desensitized to epinephrine.

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PLATELETS NORMALLY circulate in a "nonactivated" state during which they fail to interact with each other or with the vessel wall. Platelet aggregation occurs as the result of several specific interactions between the platelet surface membrane and substances in plasma. The first of these is the binding of an agonist, such as epinephrine, adenosine diphosphate (ADP), or thrombin, to discrete receptors on the platelet surface.[1] This interaction in turn triggers intracellular events that ultimately lead to the exposure of fibrinogen receptors located on the platelet membrane glycoprotein IIb-IIIa complex.[2-4] The intracellular events involved in this process are poorly understood. However, it appears that exposure of platelet fibrinogen receptors is followed almost immediately by the binding of fibrinogen to the platelet surface and by platelet aggregation. Extracellular Ca is known to be a necessary cofactor for these processes.[5,6] Studies with monoclonal antibodies specific for the IIb-IIIa complex indicate there are approximately 45,000 copies of the IIb-IIIa complex per platelet.[7-10] Since the number of fibrinogen receptors is similar, it appears that one fibrinogen molecule binds to each IIb-IIIa complex.

In pharmacologic terms, the interaction of epinephrine with human platelets has been well studied. Each platelet contains about 300 specific binding sites for epinephrine. These receptors have been partially purified and characterized as belonging to the α_2-adrenergic subclass.[11] In the presence of extracellular Ca, epinephrine can stimulate fibrinogen binding and platelet aggregation without necessarily causing platelet secretion.[12] However, as is the case with other so-called "weak agonists," platelet secretion in response to epinephrine takes place only after initial aggregation. In addition to aggregation, epinephrine stimulates the interaction of the α_2-receptor with G_i, a heterotrimeric guanine nucleotide binding protein that inhibits adenylate cyclase, leading to a decrease in the elevated cAMP levels of platelets exposed to forskolin or PGE1.[13] One curious effect of prolonged occupancy of the α_2-receptor with epinephrine is a time-dependent decrease or "desensitization" of subsequent aggregation responses to epinephrine.[14-16] Desensitization is observed when unstirred platelets are incubated for at least several minutes with epinephrine before being stirred to initiate the aggregation response. Recently, we showed that desensitization is not due to an abnormality in either the number or affinity of α_2-receptors on the platelet surface.[17] In that study, we also found that desensitization is not

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accompanied by an uncoupling of those receptors from adenylate cyclase.

The purpose of the present study was to examine the desensitization phenomenon in greater detail in order to clarify the relationships between occupancy of platelet α_2-receptors by epinephrine and several responses to receptor occupancy: Fibrinogen receptor induction, fibrinogen binding, and platelet aggregation. We focused particular attention on the role of extracellular Ca because this divalent cation is required for fibrinogen binding and for all subsequent functional responses of the platelet to epinephrine. ^{125}I-Fibrinogen was used to quantitate fibrinogen binding to platelets, and a radiolabeled monoclonal antibody, PAC-1, was used to monitor induction of the platelet fibrinogen receptor. This antibody has been shown to bind to IIb-IIIa on intact platelets at or near the platelet fibrinogen receptor, but only after the platelets have been activated.[18] These studies will demonstrate that (1) extracellular Ca is a cofactor for epinephrine-induced platelet aggregation largely because it is required for fibrinogen receptor expression; and (2) desensitization of the aggregation response to epinephrine is not explained by an impairment of either fibrinogen receptor expression or fibrinogen binding.

MATERIALS AND METHODS

Preparation of platelets. Blood was obtained from healthy donors and anticoagulated with either 1/10 vol of 0.13 mol/L sodium citrate for studies in plasma or with 1/6 vol of ACD (65 mmol/L citric acid; 56 mmol/L sodium citrate; 104 mmol/L dextrose) for studies with gel-filtered platelets. Platelet-rich plasma was obtained by differential centrifugation, and where indicated, incubated for 20 minutes at 22 °C with 1 mmol/L aspirin to inhibit platelet cyclooxygenase.[19] To obtain gel-filtered platelets, 1 μmol/L PGE$_1$ (Sigma, St Louis) was added to the platelet-rich plasma and the platelets were passed over a column of Sepharose 2B (Pharmacia Fine Chemicals) using an elution buffer containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl$_2$, 5.6 mmol/L glucose, 1 mg/mL bovine serum albumin, 3.3 mmol/L NaH$_2$PO$_4$, and 20 mmol/L HEPES, pH 7.4. This buffer contains approximately 10 μmol/L free Ca.

Platelet aggregation studies. As indicated in the text and the figure legends, unstirred platelet-rich plasma or gel-filtered platelets were preincubated at 22 °C under a variety of conditions for up to 90 minutes. Aggregation was then initiated at 37 °C in an aggregometer (Chronolog Corp, Broomall, PA) by adding a stir bar to the aggregation cuvette and stirring at 1,000 rpm. The aggregation of gel-filtered platelets required the addition of 100 μg/mL fibrinogen (Kabi, Stockholm).[5] Aggregation was monitored as a change in light transmittance and is presented either as the initial rate or as the percent change in light transmittance three minutes after adding the stir bar.[17]

Binding of ^{125}I-PAC-1 and ^{125}I-fibrinogen to platelets. The murine monoclonal antibody, PAC-1, and human fibrinogen (Kabi) were purified and radiolabeled with ^{125}I, and binding studies were carried out as described previously.[5,18] Briefly, gel-filtered platelets were suspended at 10^8/mL and incubated at 22 °C without stirring for up to 90 minutes with epinephrine. Then ^{125}I-PAC-1 (1 to 95 μg/mL) or ^{125}I-fibrinogen (10 to 1800 μg/mL) were added to 0.5-mL aliquots of the platelet suspension and incubated an additional 15 minutes at 22 °C. Platelet-bound PAC-1 was then separated from unbound antibody by filtration on 0.45-μm nitrocellulose filters. Platelet-bound ^{125}I-fibrinogen was separated from unbound fibrinogen by centrifugation of the platelets through 0.5 mL silicone oil in a microcentrifuge. Platelets on the dried filters or in the cell pellet were counted for ^{125}I in a gamma counter. Nonspecific binding of either ligand was defined as binding in the presence of 10 mmol/L EDTA.[3] In the case of PAC-1, this represented binding to the filters themselves and accounted for <5% of total binding. In the case of fibrinogen, nonspecific binding was a linear function of platelet count and represented unbound fibrinogen trapped in the extracellular volume of the platelet pellet. Binding data are presented as specific binding, defined at single-ligand concentrations as total binding minus nonspecific binding. In experiments in which a range of ligand concentrations were studied, specific binding, maximal binding, and binding constants for PAC-1 and fibrinogen were computed by nonlinear regression analysis using an equation describing reversible binding to a single class of binding sites.[18]

Unless specified otherwise, the binding studies were conducted in the standard gel-filtration buffer containing 10 μmol/L free Ca and 1 mmol/L MgCl$_2$. However, in some studies, the free Ca concentration in the gel filtration buffer was varied from 10^{-9} to 10^{-3} mol/L by the addition of CaCl$_2$ and EGTA.[20] Free Ca concentrations above 10^{-7} mol/L were verified with a Ca-selective electrode. In some experiments, MgCl$_2$ was omitted from the gel-filtration elution buffer but added to the platelet suspensions just before the binding studies.

RESULTS

The use of PAC-1 to monitor induction of the fibrinogen receptor. As reported previously, both fibrinogen and the monoclonal antibody, PAC-1, bind to the IIb-IIIa complex on the surface of intact platelets, but only after the platelets have been activated by an agonist.[2-5,18] Furthermore, PAC-1 inhibits platelet aggregation, suggesting that its binding site on the IIb-IIIa complex is located at or near the binding site for fibrinogen.[18] As part of the present studies, we examined the competition between PAC-1 and fibrinogen for binding sites on epinephrine-stimulated platelets. Unlabeled PAC-1 inhibited the binding of ^{125}I-fibrinogen in a dose-dependent manner (Fig 1). Inhibition was half maximal at about 10 nmol/L PAC-1, a value similar to the apparent Kd for PAC-1 binding to platelets (5 nmol/L).[18] Conversely, unlabeled fibrinogen competitively inhibited the binding of ^{125}I-PAC-1 to stimulated platelets (Fig 2). The Ki for fibrinogen was 200 nmol/L, similar to the Kd that others have obtained for fibrinogen binding to platelets.[4,5] Thus, the binding site for PAC-1 on platelets appears to be close to, if not identical with, the receptor for fibrinogen. Accordingly, PAC-1 was used as a specific probe to monitor induction of the platelet fibrinogen receptor by epinephrine, and ^{125}I-fibrinogen was used to monitor the end result of receptor expression, fibrinogen binding. As previously reported, the maximum number of binding sites for PAC-1 on stimulated platelets is, depending on the agonist, two- to threefold less than the maximum number of binding sites for fibrinogen. Since PAC-1 is an IgM, this discordance was attributed to the multivalent nature and size of the antibody. This conclusion is supported by the observation in the present studies that PAC-1 and fibrinogen binding are mutually competitive.

Effect of extracellular Ca and Mg on epinephrine-induced fibrinogen receptor expression and fibrinogen binding. Previous studies have demonstrated that extracellular Ca and/or Mg in the 0.1 to 1.0-mmol/L range is required for

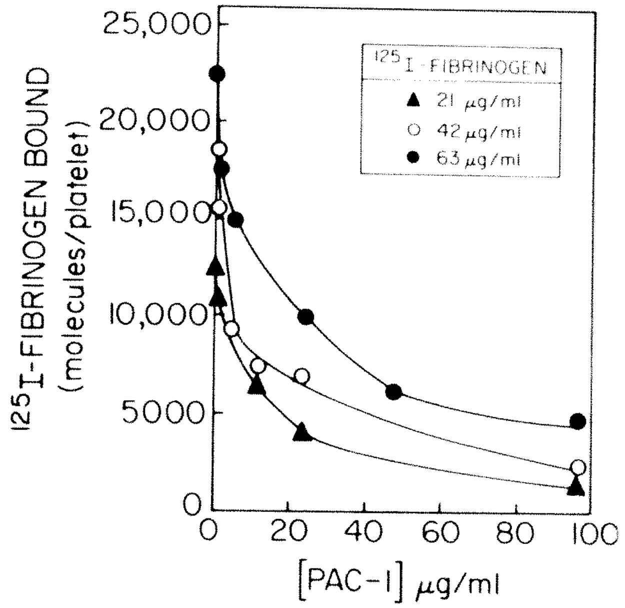

Fig 1. Inhibition of ^{125}I-fibrinogen binding by PAC-1. Gel-filtered platelets were incubated for one minute with 10 μmol/L epinephrine, at which time the platelets were added to tubes containing 21, 42 or 63 μg/mL ^{125}I-fibrinogen and 0 to 95 μg/mL PAC-1. After 15 minutes at 22 °C, platelet-bound fibrinogen was separated from unbound fibrinogen and the amount of fibrinogen bound to the platelets was determined as described in Materials and Methods. This experiment is representative of three so performed.

maximal fibrinogen binding and platelet aggregation.[2-6] In addition, the secretory response of platelets to epinephrine is entirely dependent on the presence of extracellular Ca.[6,21,22] Therefore, before investigating the function of the fibrinogen receptor during platelet desensitization to epinephrine, we examined in detail the divalent cation requirement for epinephrine-induced fibrinogen receptor expression. Gel-filtered platelets were suspended at 22 °C and pH 7.4 in an EGTA buffer at free Ca concentrations ranging from 10^{-3} to 10^{-9} mol/L. Although the membrane-bound IIb-IIIa complex is disrupted by exposure to low Ca concentrations at 37 °C and therefore fails to bind fibrinogen, the complex remains intact after exposure to low Ca concentrations at 22 °C.[23-25] The platelets were stimulated for five minutes with 10 μmol/L epinephrine, then ^{125}I-PAC-1 or ^{125}I-fibrinogen was added, and the amount of antibody or fibrinogen bound to the platelets was determined. Both PAC-1 and fibrinogen binding were dependent on the presence of extracellular Ca. PAC-1 binding was half-maximal at 30 μmol/L free Ca and maximal at 300 μmol/L Ca (Fig 3A). Fibrinogen binding was half-maximal at 100 μmol/L and maximal at 1 mmol/L Ca. This slight difference between PAC-1 and fibrinogen binding was observed consistently. It could not be accounted for by contamination of the ligand preparations with Ca, since the free Ca concentration of the final incubation buffer was not affected by the addition of either ligand. In the absence of Ca, magnesium, even at a concentration of 1 mmol/L, failed to support PAC-1 or fibrinogen binding (Figs 3B, 3C, and 3D). However, the Ca dose-response curves for both ligands were shifted to the left by Mg. At 1 mmol/L Mg, both PAC-1 and fibrinogen binding were half-maximal at 1 μmol/L Ca and maximal at 10 μmol/L Ca—a decrease in the Ca requirement by approximately two orders of magnitude (Fig 3D). Thus, the previously noted extracellular Ca requirement for fibrinogen binding appears

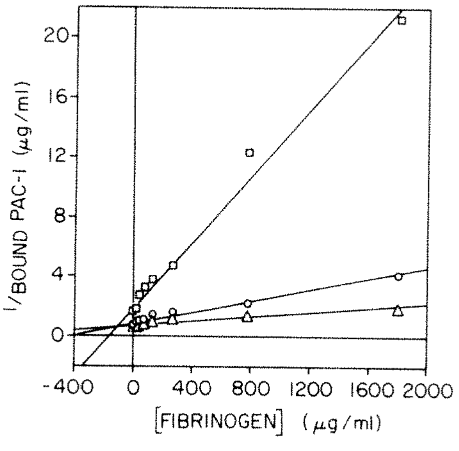

Fig 2. Inhibition of ^{125}I-PAC-1 binding by fibrinogen. Gel-filtered platelets were incubated for one minute with 10 μmol/L epinephrine and then added to tubes containing 0 to 1800 μg/mL fibrinogen and 2.8 μg/mL PAC-1 (squares), 6.3 μg/mL PAC-1 (circles), or 9.7 μg/mL PAC-1 (triangles). After 15 mintues at 22 °C, the amount of PAC-1 bound to the platelets was determined. In this Dixon plot, the regression lines for the three sets of data intersect above the x-axis, and the data are compatible with a competitive type of inhibition. This experiment is representative of three so performed.

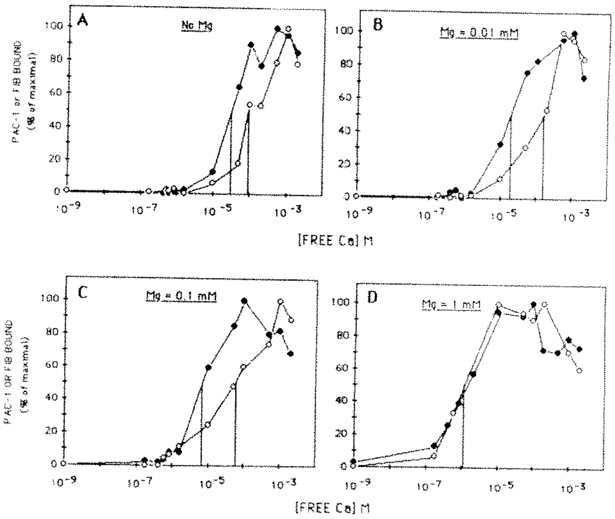

Fig 3. Effect of Ca and Mg on the binding of PAC-1 and fibrinogen to epinephrine-stimulated platelets. Gel-filtered platelets were suspended at extracellular free Ca ranging from 10^{-9} to 10^{-3} mol/L in the presence of (A) no $MgCl_2$; (B) 0.01 mmol/L $MgCl_2$; (C) 0.1 mmol/L $MgCl_2$; or (D) 1 mmol/L $MgCl_2$. The platelets were then stimulated with 10 μmol/L epinephrine for five minutes and the amounts of ^{125}I-PAC-1 (closed circles) and ^{125}I-fibrinogen (open circles) bound to the platelets were determined. The PAC-1 concentration was 12.5 μg/mL and the fibrinogen was 85 μg/mL. The vertical lines indicate the free CA concentrations at which half-maximal binding was observed. This experiment is representative of three so performed.

to reflect a Ca requirement for induction of the fibrinogen receptor. Mg reduces but does not eliminate the need for Ca. Therefore, in the desensitization experiments described below, PAC-1 and fibrinogen binding were examined at optimal concentrations of extracellular divalent cations, 10 μmol/L free Ca and 1 mmol/L Mg.

Fibrinogen binding and platelet aggregation do not occur at acid extracellular pH.[26,27] This effect of hydrogen ions also appears to be at the level of fibrinogen receptor induction because the binding of PAC-1 and fibrinogen to epinephrine-stimulated platelets was inhibited in parallel at a pH of 6.5 and below.

Induction of the fibrinogen receptor on platelets desensitized to epinephrine. The binding of epinephrine to α_2-adrenergic receptors stimulates platelet aggregation, but only if the platelets are stirred. However, after prolonged (more than five to ten minutes) incubation of unstirred platelets with epinephrine, the aggregation response is reduced when the platelets are subsequently stirred.[14-17] An example of this desensitization is shown in Fig 4. In this experiment, unstirred platelet-rich plasma was treated with 1 mmol/L aspirin to inhibit cyclooxygenase and then incubated at 22 °C with epinephrine for 90 minutes. Afterward, the sample was stirred and platelet aggregation measured. Compared to the control platelets stimulated with epinephrine at 90 minutes, the platelets that had been preincubated with epinephrine exhibited an approximate 70% reduction in their aggregation response (Fig 4). On light microscopy, the desensitized platelets formed smaller aggregates than control platelets. This reduced response could not be attributed to a decrease in epinephrine receptor occupancy because (1) we have previously shown that receptor binding is not substantially altered in desensitized platelets,[17] and (2) the addition of more epinephrine to the desensitized platelets at 90 minutes did not increase the aggregation response (Fig 4). Desensitization could also not be attributed to some component of the platelet release reaction, since it was observed both in the presence and absence of aspirin. Furthermore, it did not require a component unique to plasma because it was observed with gel-filtered platelets as well as with platelets in plasma.

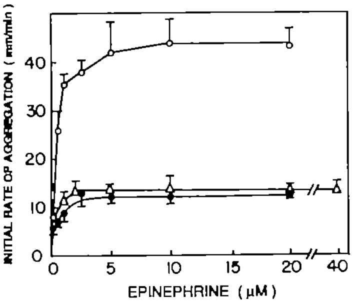

Fig 4. Desensitization of platelets to epinephrine. Aliquots of aspirin-treated platelets in plasma were incubated without stirring for 90 minutes at 22 °C. At that time, the platelets were added to aggregation cuvettes at 37 °C and aggregation was initiated by adding a stir bar. The initial rate of aggregation is arbitrarily expressed as mm/min of the aggregation trace. Open circles represent control platelets to which the epinephrine was added after the 90-minute incubation and one minute before stirring; closed circles, epinephrine added to the platelets at the very beginning of the 90-minute incubation; open triangles, epinephrine added at the indicated concentrations, both at the beginning and at the end of the 90-minute incubation. Data represent the mean ± SEM of three experiments.

Inasmuch as aggregation requires the binding of fibrinogen to its receptor, we wondered whether desensitized platelets would exhibit a reduction in the number of fibrinogen receptors expressed on their surface. To test this possibility, aspirin-treated gel-filtered platelets were pre-incubated with 10 μmol/L epinephrine for up to 90 minutes, ^{125}I-PAC-1 was added, and the amount of antibody bound to the platelets was determined. Epinephrine induced an immediate increase in PAC-1 binding, and this binding was sustained, even if the platelets were incubated with the epinephrine for 90 minutes before the addition of the ^{125}I-PAC-1 (Fig 5A). Moreover, compared to control platelets stimulated with epinephrine

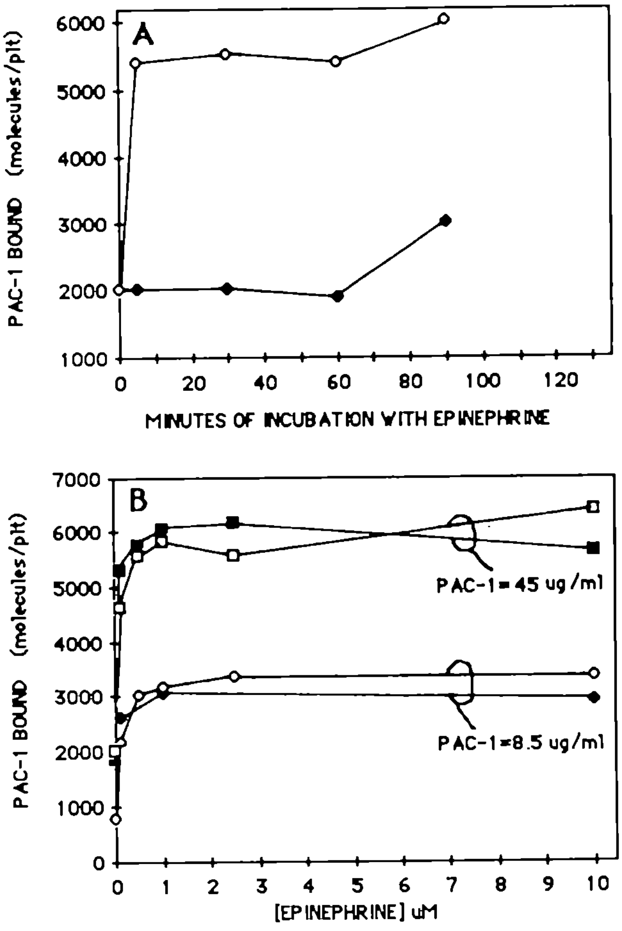

Fig 5. ^{125}I-PAC-1 binding to epinephrine-stimulated platelets. (A) Aspirin-treated gel-filtered platelets were incubated without an agonist (closed circles) or with 10 μmol/L epinephrine (open circles) for the indicated periods of time. Then 18 μg/mL ^{125}I-PAC-1 was added and the amount of PAC-1 bound to the platelets was determined. (B) Platelets were incubated with epinephrine for the entire 90 minutes (closed symbols) or for just one minute (open symbols). Then 8.5 μg/mL ^{125}I-PAC-1 (circles) or 45 μg/mL ^{125}I-PAC-1 (squares) was added, and the amount of PAC-1 bound to the platelets was determined. This experiment is representative of five so performed.

only at the end of the 90-minute period, desensitized platelets demonstrated normal PAC-1 binding over a range of epinephrine concentrations (0.1 to 10 μmol/L) (Fig 5B). The persistence of fibrinogen receptor expression in desensitized platelets was not due to an irreversible modification of this receptor because >95% of the PAC-1 binding to platelets could be prevented, even after prolonged incubations with epinephrine, by displacing the agonist from the platelet with either 10 μmol/L phentolamine or yohimbine. Thus, desensitization of the aggregation response to epinephrine cannot be explained by a reduction in the level of fibrinogen receptor expression.

Binding of fibrinogen to platelets desensitized to epinephrine. Next, we examined the effect of prolonged platelet exposure to epinephrine on fibrinogen binding itself. Aspirin-treated gel-filtered platelets were incubated for 50 minutes at 22 °C with 10 μmol/L epinephrine. ^{125}I-fibrinogen (10 to 1,800 μg/mL) was then added, and specific fibrinogen binding measured. In four experiments, unstimulated platelets incubated for 50 minutes bound small amounts of fibrinogen (3,726 ± 411 molecules/platelet; Kd = 205 ± 85 nmol/L). In contrast, control platelets incubated for 50 minutes and then exposed to epinephrine just before the addition of ^{125}I-fibrinogen bound 20,533 ± 5,915 fibrinogen molecules per platelet with a Kd of 186 ± 75 nmol/L. Desensitized platelets bound fibrinogen to an extent and with an affinity similar to the acutely stimulated control platelets (18,820 ± 3,984 molecules/platelet; Kd = 221 ± 49 nmol/L). Thus, desensitization of the aggregation response to epinephrine cannot be explained by a reduction in fibrinogen binding to the desensitized platelets.

Fibrinogen initially binds reversibly to platelets, but over 10 to 60 minutes its binding becomes progressively irreversible.[28,29] For example, when platelets were incubated with epinephrine and ^{125}I-fibrinogen for only five minutes, 90% of the bound fibrinogen could be removed by adding 10 mmol/L EDTA to chelate extracellular Ca and Mg (data not shown). In contrast, when platelets were incubated with epinephrine and fibrinogen for 60 minutes, only 39% of the bound fibrinogen could be removed. It should be noted that in our desensitization experiments, gel-filtered platelets had been preincubated with epinephrine for 50 minutes before the addition of ^{125}I-fibrinogen. Under these conditions, 96% of the fibrinogen that bound to desensitized platelets was removed with EDTA. Since the binding of fibrinogen to desensitized platelets is readily reversible, desensitization cannot be attributed to irreversible fibrinogen binding.

Although desensitization does not appear to be due to an abnormality of fibrinogen receptor expression, it is still possible that prolonged expression of the receptor on unstirred platelets leads in some way to a reduction of the subsequent aggregation response. This possibility was addressed in two different experiments. In the first, aspirin-treated platelet-rich plasma was incubated at 22 °C with 5 μmol/L epinephrine and 2.5 mmol/L EDTA for 50 minutes. The EDTA was added to prevent fibrinogen receptor expression and fibrinogen binding, and it does not interfere with the binding of the epinephrine to platelets.[30] After 50 minutes, 2.5 mmol/L $CaCl_2$ was added and platelet aggregation studies were carried out. Whether expressed in terms of the initial rate or maximal extent of aggregation, the platelets that had been incubated with epinephrine and EDTA aggregated significantly better than the "desensitized" platelets that had been incubated with epinephrine alone ($P < 0.01$) (Fig 6). In the second experiment, unstirred gel-filtered platelets were incubated for 50 minutes with 10 μmol/L epinephrine in the presence or absence of 100 μg/mL fibrinogen. At 50 minutes, fibrinogen was added to those samples lacking it, and the platelets were stirred and aggregation measured. Desensitization of the aggregation response was not affected significantly by the presence of fibrinogen during the initial 50-minute incubation period (Fig 7). These experiments suggest that fibrinogen binding, per se, plays little or no role in the mechanism of desensitization of the subsequent aggregation response. On the other hand, some role for prolonged expression of the fibrinogen receptor in the desensitization process cannot be excluded.

DISCUSSION

Platelet aggregation represents the functional end-response of a sequence of reactions that takes place largely at the platelet surface membrane. This includes (1) the binding of an agonist to its receptor; (2) transmission of a signal(s) from the receptor to the membrane glycoprotein IIb-IIIa complex; (3) a modification of the IIb-IIIa complex that converts it into a functional fibrinogen receptor; and (4) the binding of fibrinogen to the IIb-IIIa complex. The availability of PAC-1, a monoclonal antibody that is specific for the fibrinogen receptor on activated platelets, has enabled us to distinguish between those factors involved in the induction of the fibrinogen receptor and those involved in fibrinogen binding. In the present study, we have examined the induction of the fibrinogen receptor by epinephrine as well as the function of this receptor during desensitization of the platelet aggregation response to epinephrine.

Platelet aggregation in response to epinephrine is dependent on extracellular Ca.[6,21,22] Moreover, platelet secretion in

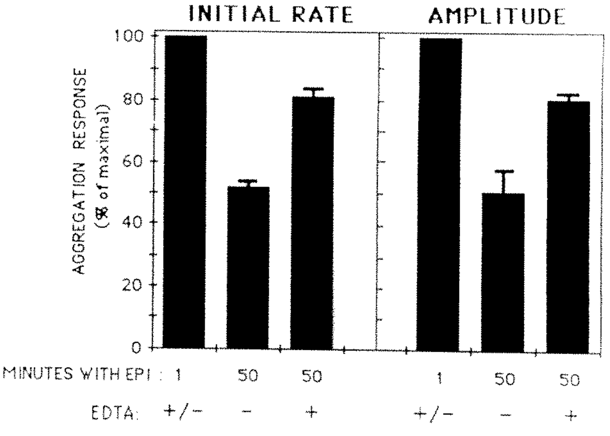

Fig 6. Effect of EDTA on platelet desensitization to epinephrine. See text for details. The aggregation responses of control platelets incubated with epinephrine for only one minute were set arbitrarily at 100%. Data for the initial rate and the maximal extent (amplitude) of aggregation represent the mean ± SEM of three experiments.

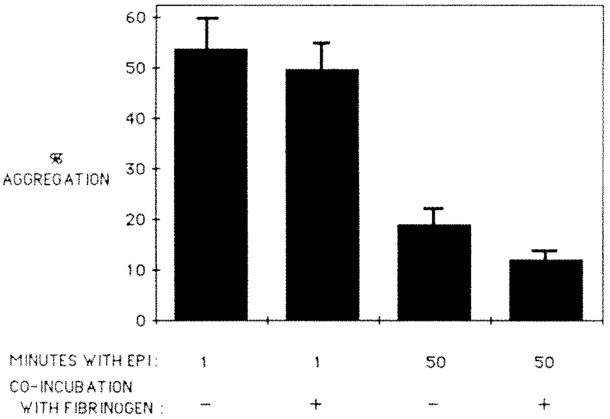

Fig 7. Effect of prolonged incubation with fibrinogen on platelet desensitization to epinephrine. Unstirred, gel-filtered platelets were incubated for 50 minutes at 22 °C, with or without 10 µmol/L epinephrine and with or without 100 µg/mL fibrinogen. At 50 minutes, epinephrine and fibrinogen were added to those samples lacking them, and one minute later, the platelets were stirred in an aggregometer. The percent aggregation three minutes after the beginning of stirring was measured. Data represent the mean ± SEM of three experiments.

response to epinephrine is dependent on an initial aggregation response.[31,32] Therefore, we first examined the role of Ca in fibrinogen receptor expression. PAC-1 binding to epinephrine-stimulated platelets was absolutely dependent upon extracellular Ca. Measured in the absence of Mg, PAC-1 binding was half-maximal at 30 µmol/L free Ca and maximal at 300 µmol/L. Thus, Ca in the millimolar-to-micromolar range is needed in order for fibrinogen receptors to be expressed. The Ca dose-response curve for fibrinogen binding was slightly to the right of the PAC-1 dose-response curve. Both curves were shifted to the left in the presence of Mg such that at 1 mmol/L Mg, both PAC-1 and fibrinogen binding were half-maximal at 1 µmol/L free Ca. Although the platelets were exposed to Ca as low as 10^{-9} mol/L, our results cannot be explained by a disruption of the membrane IIb-IIIa complex which is known to occur with Ca depletion at 37 °C. In the present experiments, platelets were exposed to low Ca at 22 °C, a temperature at which the membrane-associated complex remains intact.[23-25]

It has been reported that either Ca or Mg in the mmol/L range can support fibrinogen binding and platelet aggregation.[5] However, the present study demonstrates that Ca is a necessary and sufficient divalent cation for full induction of the fibrinogen receptor and for maximal fibrinogen binding. Mg can play a permissive role but is not an effective substitute for Ca. This conclusion is consistent with previous aggregation experiments and with studies of fibrinogen binding to platelet membranes, both of which have demonstrated a differential role for the two divalent cations.[6,33] Other reports suggesting that Mg alone can support aggregation have used buffers that contained "no added Ca." Such buffers probably contained at least 1 to 10 µmol/L residual Ca, enough to support fibrinogen receptor expression in the presence of Mg (Fig 3).

The present study does not clarify how extracellular Ca is involved in epinephrine-induced fibrinogen receptor expression. Induction of the receptor by ADP and thrombin is also Ca-dependent.[18] In theory, Ca might be involved in the agonist binding step, in the signal transmission step that links agonist binding to exposure of the fibrinogen receptor on IIb-IIIa, or directly at the level of IIb-IIIa. The first possibility is excluded by the fact that extracellular Ca is not required for optimal binding of α_2-adrenergic agonists.[34] The Ca requirement could be explained by an agonist-induced influx of extracellular Ca, which could in turn initiate a Ca-dependent biochemical process within the platelet that triggers fibrinogen receptor expression. For example, ADP and thrombin stimulate an increase in cytoplasmic free Ca (measured by quin 2) that is greater in the presence of extracellular Ca than in its absence.[35] Furthermore, both agonists stimulate calmodulin– and protein kinase C–dependent protein phosphorylation.[36] On the other hand, epinephrine is a potent stimulator of fibrinogen receptor expression despite the fact that it does not cause a bulk increase in cytoplasmic Ca or protein phosphorylation in aspirin-treated platelets.[35,37] Thus, if epinephrine does cause an influx of extracellular Ca, this would have to represent a quantitatively minor but strategically localized Ca pool that is involved in fibrinogen receptor exposure. Indeed, using aequorin rather than the fluorescent Ca probe quin 2 to measure cytoplasmic free Ca, Ware and coworkers have detected epinephrine-induced Ca transients that could reflect such a localized pool.[35] Similarly, Sweat et al have found that epinephrine causes changes in chlortetracycline fluorescence in indomethacin-treated platelets.[38] Alternatively, a possible direct effect of Ca on the conformation of IIb and IIIa must be considered because extracellular Ca appears to bind directly to these glycoproteins.[6] However, further assessment of the effect of Ca on IIb-IIIa structure will require work with purified, functional IIb-IIIa complexes reconstituted into artificial membranes.[39]

Although we have emphasized a potential direct interaction between extracellular Ca and the platelet, it is also possible that additional interactions between Ca or Mg and fibrinogen are important for the binding of this ligand to IIb-IIIa. We found that the Ca dose-response curves for PAC-1 and fibrinogen binding were not superimposable in the absence of Mg (Fig 3A). Several classes of divalent cation binding sites on fibrinogen have been demonstrated,[40] but their functional significance with respect to fibrinogen binding remains to be determined.

In most experimental situations, a close relationship has been demonstrated between the extent of fibrinogen binding and the extent of platelet aggregation.[2,3,5] However, the precise mechanism of platelet-to-platelet interaction remains unknown. The dimeric structure of fibrinogen suggests that the molecule functions as an intercellular bridge by binding to fibrinogen receptors on adjacent platelets. Peerschke and coworkers have demonstrated that platelets activated by an agonist and then fixed with formalin are capable of binding fibrinogen but cannot aggregate.[41] On this basis they have proposed that membrane events distal to fibrinogen binding are necessary for maximal platelet aggregation. Such events might include the interaction of the fibrinogen receptor with components of the membrane cytoskeleton,[42,43] the lateral movement of the fibrinogen receptor within the plane of the membrane, and receptor clustering.[44-46]

Our studies also indicate that the extent of fibrinogen binding does not necessarily determine the extent of platelet aggregation. When unstirred platelets were exposed to epinephrine for up to 90 minutes, they bound the same amount of PAC-1 and fibrinogen as did control platelets exposed only acutely to epinephrine. Despite maximal fibrinogen receptor expression and fibrinogen binding, these desensitized platelets exhibited a 70% reduction in their aggregation response as measured by changes in light transmittance. This reflected a decrease in the size of the aggregates. Thus, desensitization resulted in a clear dissociation between fibrinogen binding and platelet aggregation. A qualitatively similar, though less marked, dissociation between fibrinogen binding and aggregation has been observed after platelet desensitization to ADP.[47]

Several possible mechanisms for desensitization of the aggregation response to epinephrine need to be considered. First, it has been suggested that "secondary," or irreversible, platelet aggregation involves the binding of thrombospondin to the platelet surface after its release from platelet α-granules.[48] Thrombospondin is unlikely to be involved in our aggregation studies because desensitization was observed even when the release reaction was prevented with aspirin. Second, prolonged occupancy of the fibrinogen receptor by fibrinogen might play a role in desensitization. However, we found that it was not necessary for fibrinogen to be added during the prolonged incubation with epinephrine in order for desensitization to be observed. We did find, however, that if EDTA were present during the incubation with epinephrine, desensitization was partially prevented. This effect of EDTA may have resulted from suppression of fibrinogen receptors, but other possible mechanisms cannot be ruled out. Although speculative, it is possible that persistent expression of fibrinogen receptors is involved in the sequence of events leading to desensitization, perhaps by limiting the lateral movement or clustering of the receptors after fibrinogen binds. Whatever the mechanism of desensitization, the current results indicate that there are platelet reactions subsequent to fibrinogen binding that determine the maximal extent of the aggregation response. These reactions are impaired when platelets are desensitized to epinephrine.

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REFERENCES

1. Shattil SJ, Bennett JS: Platelets and their membranes in hemostasis: Physiology and pathophysiology. Ann Intern Med 94:108, 1981

2. Bennett JS: The platelet fibrinogen interaction, in George JN, Nurden AT, Phillips DR (eds): Platelet Membrane Glycoproteins. New York, Plenum, 1985, p 139

3. Peerschke EIB: The platelet fibrinogen receptor. Semin Hematol 22:241, 1985

4. Plow EF, Marguerie GA: Induction of the fibrinogen receptor on human platelets by epinephrine and the combination of epinephrine and ADP. J Biol Chem 22:10971, 1980

5. Bennett JS, Vilaire G: Exposure of platelet fibrinogen receptors by ADP and epinephrine. J Clin Invest 64:1393, 1979

6. Brass LF, Shattil SJ: Identification and function of the high affinity binding sites for Ca2+ on the surface of platelets. J Clin Invest 73:626, 1984

7. Bennett JS, Hoxie JA, Leitman SF, Vilaire G, Cines DB: Inhibition of fibrinogen binding to stimulated platelets by a monoclonal antibody. Proc Natl Acad Sci USA 80:2417, 1983

8. Coller BS, Peerschke EIB, Scudder LE, Sullivan CA: A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and IIIa. J Clin Invest 72:325, 1983

9. DiMinno G, Thiagarajan P, Perussia B, Martinez J, Shapiro S, Trinchieri G, Murphy S: Exposure of platelet fibrinogen-binding sites by collagen, arachidonic acid, and ADP: Inhibition by a monoclonal antibody to the glycoprotein IIb-IIIa complex. Blood 61:140, 1983

10. Pidard D, Montgomery RR, Bennett JS, Kunicki TJ: Interaction of AP-2, a monoclonal antibody specific for the human platelet glycoprotein IIb-IIIa complex, with intact platelets. J Biol Chem 258:12582, 1983

11. Regan JW, DeMarinis RM, Caron MG, Lefkowitz RJ: Identification of the subunit-binding site of α_2-adrenergic receptors using [3H]phenoxybenzamine. J Biol Chem 259:7864, 1984

12. Bennett JS, Vilaire G, Burch JW: A role for prostaglandins and thromboxanes in the exposure of platelet fibrinogen receptors. J Clin Invest 68:981, 1981

13. Katada T, Bokoch GM, Northup JK, Ui M, Gilman AG: The inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. Properties and function of the purified protein. J Biol Chem 259:3568, 1984

14. O'Brien JR: Variability in the aggregation of human platelets by epinephrine. Nature 202:1188, 1964

15. Cooper B, Handin RI, Young LH, Alexander RW: Agonist regulation of the human platelet alpha-adrenergic receptor. Nature 274:703, 1978

16. Hollister AS, Fitzgerald GA, Nadeau JHJ, Robertson D: Acute reduction in human platelet α_2-adrenoreceptor affinity for agonist by endogenous and exogenous catecholamines. J Clin Invest 72:1498, 1983

17. Motulsky HJ, Shattil SJ, Ferry N, Rozansky D, Insel PA: Desensitization of epinephrine-initiated platelet aggregation does not alter binding to the α_2-adrenergic receptor or receptor coupling to adenylate cyclase. Mol Pharmacol 29:1, 1986

18. Shattil SJ, Hoxie JA, Cunningham M, Brass LF: Changes in the platelet membrane glycoprotein IIb-IIIa complex during platelet activation. J Biol Chem 260:11107, 1985

19. Burch JW, Baenziger NL, Stanford N, Majerus PW: Sensitivity of fatty acid cyclooxygenase from human aorta to acetylation by aspirin. Proc Natl Acad Sci USA 75:5181, 1978

20. Brass LF, Shattil SJ: Changes in surface-bound and exchangeable calcium during platelet activation. J Biol Chem 257:14000, 1982

21. Scrutton MC, Egan CM: Divalent cation requirements for aggregation of human blood platelets and the role of anticoagulant. Thromb Res 14:713, 1979

22. Lages B, Weiss HJ: Dependence of human platelet functional responses on divalent cations: Aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and effects of chelating agents. Thromb Haemost 45:173, 1981

23. Shattil SJ, Brass LF, Bennett JS, Pandhi P. Biochemical and functional consequences of dissociation of the platelet membrane glycoprotein IIb-IIIa complex. Blood 66.92, 1985

24. Fitzgerald LA, Phillips DR Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. J Biol Chem 260:11366, 1985

25. Pidard D, Didry D, Kunicki TJ, Nurden AT: Temperature-dependent effects of EDTA on the membrane glycoprotein IIb-IIIa complex and platelet aggregability. Blood 67:604, 1986

26 Marguerie GA, Plow EF: Interaction of fibrinogen with its platelet receptor: Kinetics and effect of pH and temperature. Biochemistry 20:1074, 1981

27. Peerschke EIB, Zucker MB, Grant RA, Egan JJ, Johnson MM: Correlation between fibrinogen binding to human platelets and platelet aggregability. Blood 55:841, 1980

28. Marguerie GA, Edgington TS, Plow EF· Interaction of fibrinogen with its platelet receptor as part of a multistep reaction in ADP-induced platelet aggregation. J Biol Chem 255:154, 1980

29. Peerschke EIB, Wainer JA: Examination of irreversible platelet-fibrinogen interactions. Am J Physiol 248·C466, 1985

30. Motulsky HJ, Shattil SJ, Insel PA: Characterization of α_2-adrenergic receptors on human platelets using [^{3}H]yohimbine. Biochem Biophys Res Commun 97 1562, 1980

31. Charo IF, Feinman RD, Detwiler TC. Interrelations of platelet aggregation and secretion. J Clin Invest 60:866, 1977

32. Huang EM, Detwiler TC: Characterization of the synergistic actions of platelet agonists. Blood 57:685, 1981

33. Phillips DR, Baughn AK: Fibrinogen binding to human platelet membranes. Identification of two steps requiring divalent cations. J Biol Chem 258:10240, 1983

34. Tsai BS, Lefkowitz RJ. Agonist-specific effects of monovalent and divalent cations on adenylate cyclase coupled alpha-adrenergic receptors in rabbit platelets. Mol Pharmacol 14:540, 1978

35. Ware JA, Johnson PC, Smith M, Salzman EW: The effect of common agonists on cytoplasmic ionized calcium concentration in platelets: Measurement with quin 2 and aequorin. J Clin Invest 77:878, 1986

36. Nishizuka Y, Takai Y, Kishimoto A, Kikkawa U, Kaibuchi K: Phospholipid turnover in hormone action. Recent Prog Horm Res 40.301, 1984

37 Kawahara Y, Yamanishi J, Tsunemitsu M, Fukuzaki H. Protein phosphorylation and diglyceride production during serotonin release induced by epinephrine plus ADP in human platelets Thromb Res 30:477, 1983

38. Sweat JD, Connolly TM, Crague EJ, Limbird LE: Evidence that Na$^+$/H$^+$ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. J Biol Chem 261:8667, 1986

39. Parise LV, Phillips DR. Platelet membrane glycoprotein IIb-IIIa complex incorporated into phospholipid vesicles. Preparation and morphology. J Biol Chem 260:1750, 1985

40 Marguerie G, Chagniel G, Suscillon M: The binding of calcium to bovine fibrinogen. Biochim Biophys Acta 490:94, 1977

41. Peerschke EI, Zucker MB: Fibrinogen receptor exposure and aggregation of human blood platelets produced by ADP and chilling. Blood 57.663, 1981

42 Phillips DR, Jennings LK, Edwards HH: Identification of membrane proteins mediating the interaction of human platelets. J Cell Biol 86 77, 1980

43. Painter RG, Ginsburg M. Conconavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. J Cell Biol 92 565, 1982

44. Polley MJ, Leung LLK, Clark FY, Nachman R: Thrombin-induced platelet membrane GPIIb and IIIa complex formation An electron microscopic study. J Exp Med 154:1058, 1981

45. Loftus JC, Albrecht RM: Redistribution of the fibrinogen receptor of human platelets after surface activation. J Cell Biol 99:822, 1984

46. Santoso S, Zimmermann U, Neppert J, Mueller-Eckhardt C: Receptor patching and capping of platelet membranes induced by monoclonal antibodies. Blood 67:343, 1986

47. Peerschke EIB: Ca2+ mobilization and fibrinogen binding of platelets refractory to adenosine diphosphate stimulation. J Lab Clin Med 106 111, 1985

48. Leung LLK: Role of thrombospondin in platelet aggregation. J Clin Invest 74:1764, 1984

Use of Surface Marker Analysis to Predict Outcome of Adult Acute Myeloblastic Leukemia

By James D. Griffin, Roger Davis, Douglas A. Nelson, Frederick R. Davey, Robert J. Mayer, Charles Schiffer, O. Ross McIntyre, and Clara D. Bloomfield

In order to investigate the clinical significance of surface antigen analysis in acute myeloblastic leukemia (AML), the blasts from 196 patients with AML were analyzed prospectively with a panel of 16 monoclonal antibodies. The antibodies were selected to identify differentiation-associated antigens of either the myeloid lineage (MY9, PM-81, AML-2-23, MY7, MCS-1, MY8, Mo1, MY1, MY4, Mo2), T cell lineage (T101, T11), B cell lineage (B1, B4) or multiple lineages [J5 (CALLA), HLA-DR]. Independent morphological review and classification by French-American-British (FAB) criteria was performed in 161 of the 196 cases. One or more myeloid surface antigens were detected on the blasts of 195 cases, while B and T cell markers were detected on 0% to 2% of cases. When both blood and marrow samples were studied on the same patient, very few differences were noted between the antigenic profiles of the paired specimens. The frequency of expression of individual myeloid antigens ranged from 91% (PM-81) to 29% (Mo2). Expression of individual antigens was found to correlate significantly with several clinical parameters including FAB classification, cytochemical staining for alpha naphthyl acetate esterase, leukocyte count, and the presence of extramedullary disease at presentation. Two myeloid antigens (MY4 and MY7) predicted for a low rate of complete remission (CR) to standard induction chemotherapy. MY4+ cases (37% of the total population) had a CR rate of 53%, while M4− cases had a CR rate of 69% ($P = .03$). MY7+ cases (57% of the total population) had a CR rate of 55% while MY7− cases had a CR rate of 73% ($P = .01$). Neither MY4 nor MY7 antigen expression was correlated with patient age. Paired combinations of antigens were also examined. The [MY4− MY7−] phenotype was exhibited by 32% of all cases and was associated with an 82% CR rate while the CR rate of all other cases was 54% ($P = .001$). The expression of three antigens (HLA-DR, MY8, Mo1) was associated with a decreased continuous complete remission ($P < .05$, median follow-up time of 19 months). Expression of MY8 antigen was also associated with decreased survival ($P = .03$). These results confirm earlier reports of antigenic heterogeneity in AML, and indicate that immunologically defined subgroups of AML patients which are of potential clinical significance can be identified.

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ACUTE MYELOBASTIC LEUKEMIA (AML) is the most common form of acute leukemia in adults. Despite considerable improvements in the rate of remission following treatment with chemotherapy,[1-4] most patients will ultimately die with relapsed leukemia. Although there is clinical, morphological, and laboratory evidence of patient heterogeneity, identification of prognostically important subgroups has proven difficult. Age greater than 60 years has been an adverse feature in most studies,[1,5-7] and some studies have identified other poor prognostic categories including a history of myelodysplastic syndrome,[7] prior chemotherapy or radiotherapy,[8] leukemic colony growth characteristics in vitro,[9-11] bleeding or infection,[12] and specific cytogenetic abnormalities.[13] The French-American-British (FAB) classification system has provided a popular and generally reproducible classification system for AML based on cell morphology.[14-16] Some investigations have identified prognostically significant groups within the FAB system,[17-19] while others have not.[20,21]

In acute lymphoblastic leukemia (ALL), certain immunologic tests have been used to augment morphological classifications, and have greatly facilitated differential diagnosis, cell lineage association, and identification of ALL patients with both good and poor prognoses.[22-26] For example, T cell ALL cases identified by rosetting with sheep erythrocytes or by surface marker analysis with anti-T cell heteroantisera or monoclonal antibodies respond poorly to standard chemotherapy regimens,[23] but respond well to more intensive chemotherapy programs.[24]

Surface marker analysis of blasts from patients with acute myeloblastic leukemia has not been extensively investigated, despite the availability of well-characterized monoclonal antibodies recognizing determinants expressed on normal and leukemic myeloid cells. This may have resulted from the difficulty in obtaining samples of leukemic cells for study from large numbers of similarly treated AML patients. Based on preliminary studies which suggested that immunologically defined subgroups of AML patients could be identified with antimyeloid monoclonal antibodies,[27-31] the Cancer and Leukemia Group B (CALGB) initiated a prospective, multiinstitutional study in 1983 to evaluate the potential ability of surface marker analysis in AML to identify prognostically important subgroups of patients. This report summarizes data obtained on the first 258 cases studied.

MATERIALS AND METHODS

Patients

All previously untreated patients receiving chemotherapy for acute myeloblastic leukemia on CALGB protocols 7921, 8221, 8321, or 8323 (protocols available from CALGB Central Office) from January 1, 1983, to December 1, 1984, were eligible for this study. Registration required the availability of fresh bone marrow or

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peripheral blood for marker analysis, and unstained smears for morphological review. Informed consent was obtained in accord with individual institutional review boards. Induction chemotherapy in all protocols consisted of cytarabine 100–200 mg/m^2 daily by continuous intravenous infusion for seven days and daunorubicin 45 mg/m^2/day (30 mg/m^2 for patients over 60 years) by rapid intravenous infusion for the first three days of treatment. A second course of induction therapy was administered as necessary. All protocols included postinduction chemotherapy administered for periods of 8 to 36 months. These regimens included cytarabine with or without other drugs. Complete remission (CR) was defined by standard CALGB criteria.[1]

Sample Handling

Aspirated bone marrow and/or peripheral blood samples containing heparin were obtained at diagnosis. Samples were shipped at ambient temperature to arrive at the central immunology laboratory (Dana-Farber Cancer Institute, Boston, MA) within 24 hours. Mononuclear cell suspensions were immediately prepared by Ficoll-Hypaque sedimentation (1.077 g/mL) and were cryopreserved in the vapor phase of liquid nitrogen in 10% dimethylsulphoxide and 20% newborn calf serum. Prior to analysis, leukemic cells were thawed in the presence of DNase I (100 μg/mL, Worthington Biochemicals) in order to minimize cell agglutination. Cytocentrifuge smears were made prior to freezing and after thawing to evaluate the percentage of blasts in each specimen. Samples with less than 40% blasts after Ficoll-Hypaque separation were considered inadequate for surface marker analyses.

Immunofluorescence Analysis

Antigen expression by leukemic cells was determined by indirect immunofluorescence and flow cytometry as previously described.[31] 5×10^5 cells were suspended in 0.1 mL of minimal essential medium containing 2.5% human AB serum to minimize nonspecific Fc receptor binding. 0.1 mL of each monoclonal antibody (as ascites) diluted 1:250 in the same medium was added to separate aliquots, and the mixture incubated at 4 °C for 30 minutes, washed twice in human serum–containing medium, and the cells further incubated at 4 °C for 30 minutes with 0.1 mL of a 1.40 dilution of fluoresceinated goat antimouse Ig (Coulter Immunology, Hialeah, FL). All monoclonal antibodies and the fluoresceinated goat antimouse immunoglobulin reagent were used at concentrations previously determined to be saturating. An IgG2a anti-Ig idiotype monoclonal (clone 89) was used as an Ig control antibody, and a nonreactive IgM monoclonal antibody was used as an IgM control antibody. Cell fluorescence was determined by cytofluorography (Ortho Diagnostics, Raritan, NJ) analyzing 10,000 cells per sample using a linear amplifier for fluorescence. Fluorescence gain was standardized daily using fixed autofluorescent chicken erythrocytes. Background fluorescence (determined by control monoclonal antibody staining) was subtracted in each case. A positive reaction was defined as 20% of leukemic cells more fluorescent than control.[31]

Monoclonal Antibodies

A panel of 16 monoclonal antibodies[22,31–46] was selected for testing on the basis of preliminary data suggesting their utility in leukemia phenotyping (Table 1). Ten antibodies identified antigens associated with normal myeloid cells, two antibodies reacted with normal B lymphocytes, two antibodies with normal T lymphocytes, and one antibody (anti–HLA-DR) was not lineage-associated. The characteristics of these antibodies and their origins are summarized in Table 1. A diagrammatic summary of the distribution of the myeloid antigens on normal marrow myeloid cells is shown in Fig 1. All antibodies were obtained from the original source (see references).

Central Review of Pathology and Cytochemistry

Unstained bone marrow and blood slides were sent to a single central laboratory (Upstate Medical Center, Syracuse, NY) for review. FAB classification was determined on Wright-Giemsa–stained smears using standard criteria.[14–16] Slides were stained for Sudan Black B (SBB), myeloperoxidase (PX), diaminobenzidine-hydroperoxidase (DAB), alpha naphthyl acetate esterase (ANAE)

Table 1. Monoclonal Antibodies Used for AML Phenotype Analysis

	Antibody	Antigen*	CD†	Antigen Expression On Normal Cells		Reference and Source
				Mature Hematopoietic Cells	Bone Marrow Cells*	
1	9-49	HLA-DR		Monocytes, B Cells	CFU-GM, BFU-E, CFU-GEMM	47
2	MY9	68kD	Cp16	Monocytes	Promyelocytes, blasts	39
3	PM-81	LNF III		Granulocytes	GPC, CFU-GM	45
4	AML-2-23	55kD		Monocytes, granulocytes	GPC, CFU-GM	43,44
5	MY7	150kD	CDw13	Monocytes, granulocytes	GPC, CFU-GM, BFU-E, CFU-GEMM	31,38
6	MCS-1	NR	CDw15	Monocytes, granulocytes	GPC	41
7	MY8	NR		Monocytes, granulocytes	GPC	31
8	Mo1	155,95kD	CD11	Monocytes, granulocytes, NK cells	GPC	40,46
9	MY4	55kD	CDw14	Monocytes	Monocytes	31
10	Mo2	55kD	CDw14	Monocytes	Monocytes	40,46
11	MY1	LNF III		Granulocytes	GPC	42
12	T101	65kD	CD5	T Cells	T Cells	48
13	T11	SRBC Receptor, 45kD	CD2	T Cells	T Cells	49
14	B1	35kD	CD20	B Cells	B Cells and Pre-B Cells	50
15	B4	94kD	CD19	B Cells	B Cells and Pre-B cells	51
16	J5	CALLA, 95kD	CD10		Granulocytes, Pre-B Cells	52

*GPC, granulocyte progenitor cells (promyelocyte, myelocyte, metamyelocyte); CFU-GM, granulocyte-monocyte colony-forming cell; BFU-E, erythroid burst-forming unit, CFU-E, erythroid colony-forming unit; LNF III, lacto-N-Fucopentaose III; NR, not reported; SRBC, sheep erythrocyte

†Cluster Designation from the 1st and 2nd International Workshops on Leucocyte Differentiation Antigens.[59]

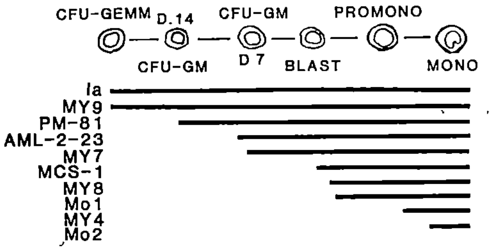

Fig 1. Diagrammatic representation of the expression of myeloid surface antigens during normal marrow myelopoiesis. The bars indicate the presence of detectable antigen on CFU-GEMM, mixed-lineage progenitor cell; day 7 and day 14 CFU-GM, granulocyte/monocyte colony forming cell; blast, myeloblasts; promonocytes; and mono, monocytes. See Table 1 for references and for information regarding expression of antigens on peripheral blood cells. In addition to the progenitor cells shown, Ia and MY9 antigens are expressed by the erythroid burst-forming cell, BFU-E.[39] None of the other antigens has been reported to react with erythroid progenitor cells.

with sodium fluoride inhibition, alpha naphthyl butyrate esterase (ANBE), periodic acid Schiff (PAS), and chloroacetate esterase (CAE) activities.[47,48]

Statistical Methods

This study was designed to correlate reactivity of monoclonal antibodies with clinical characteristics and to assess their value as prognostic indicators. Survival was measured from date of entry onto the clinical study and included all cases regardless of remission induction result. Continuous complete remission (CCR) rate was computed only for patients who achieved CR and was measured from the date CR was achieved. A remission death was treated as a censored observation for this endpoint. Comparisons involving survival and CCR were based on the log-rank test and one-year rates were estimated by the method of Kaplan and Meier.[49]

Comparisons of relationships between marker studies and patient characteristics were based on Fisher's exact test.[50] Comparisons of response by marker reactivity stratified by age were assessed using an exact test for the analysis of combinations of 2 x 2 tables [51] All *P* values reported are two-sided Because of the inherent difficulties of the "multiple comparisons" problem,[52] caution must be used in interpreting the reported *P* values

RESULTS

Patient Population

Two hundred fifty-eight patients were registered onto this study and had samples submitted for surface marker analysis. Samples were insufficient for analysis in 51 cases [insufficient cell number, 25 cases; low blast percentage (less than 40%), four; damaged sample, five; no viable cells, 17] An additional five cases never received treatment and thus provided no clinical data. The remaining 202 cases were analyzed. Six cases did not meet criteria for acute nonlymphoblastic leukemia (ANLL) on central pathology review and were eliminated from this analysis. The remaining 196 cases had surface marker analysis and form the basis for this report.

The patients' clinical characteristics at time of diagnosis are shown in Table 2. The patients had a median age of 51.5 years (range 15 to 83). The overall complete remission rate was 63%

Table 2. Patient Characteristics (N = 196)

Characteristic	Number
Age (years)	
15–20	11
20–39	48
40–59	74
60–83	63
Sex	
Female	99
Male	97
Extramedullary disease	
Central nervous system	11
Peripheral nervous system	3
Liver	32
Spleen	32
Nodes	29
Mediastinal	2
Skin/gums	37
Other	7
Any extramedullary disease	88
WBC (x 10^9/L)	
Under 4	36
4–9	26
10–19	31
20–49	50
50–99	25
100+	27
Auer rods present	106/172
Peroxidase reactivity	
Positive (≥3% of blasts)	148
Negative	16
Not Done	32
Alpha naphthyl acetate esterase reactivity	
Positive (≥20% blasts)	104
Negative	66
Not Done	26
Treatment study	
7921	6
8221	106
8321	77
8323	7

Morphologic Classification

One hundred sixty-one of the 196 cases (82.1%) with complete surface marker analysis were reviewed by the CALGB central morphology review panel and classified using FAB criteria.[14–16] The complete remission rates, continuous complete remission rate at 1 year, and survival at 1 year in relation to FAB classification are shown in Table 3

Surface Antigens

Eighty-two percent of samples analyzed were bone marrow, and 18% were blood (blood was analyzed only when marrow was not available). Availability of cells limited the number of antibodies tested in less than 10% of cases. AML-2-23, PM-81, and B4 antibodies were added to the panel during the course of the study, and therefore fewer cases were tested with these reagents. Myeloid surface antigens were detected on the blasts of all but one of 196 cases (an FAB M4). Lymphoid markers (B cell, B1 or B4; T

Table 3. Relationship of FAB Classification to Complete Remission Rate, Duration of Complete Remission and Survival

FAB Group	Response Rate ($P = 46$)*	1-Year Continuous Complete Remission (% ± SE) ($P = 003$)†	1-Year Survival Rate (% ± SE) ($P = 24$)†
M1	15/22 (68%)	76 ± 12	64 ± 10
M2	18/30 (60%)	80 ± 13	49 ± 10
M3	10/11 (91%)	73 ± 16	80 ± 13
M3V	1/3 (33%)	100 (1/1)	67 ± 27
M4	37/61 (61%)	42 ± 9	38 ± 7
M4E	7/9 (78%)	100	75 ± 15
M5A	6/9 (67%)	20 ± 18	26 ± 16
M5B	9/13 (69%)	62 ± 17	59 ± 14
M6	1/3 (33%)	0 (0/1)	0 (0/3)
All Cases	104/161 (65%)	58 ± 6	49 ± 4

*P value based on exact test.

†P value derived from a log-rank test comparing overall equality of end points (duration of complete remission or survival) across all FAB groups.

‡All cases

cell, T101 or T11; or CALLA) were detected on 2% or less of cases, and these patients will be described separately below.

Comparison of Surface Antigens Expressed by Blasts in Paired Samples From Peripheral Blood and Bone Marrow

Since both blood and bone marrow samples were analyzed in this study, a pilot study of 31 cases was performed in which cells obtained from both blood and marrow from the same patient were studied simultaneously for surface antigen expression in order to determine the comparability of these two sources of leukemic cells. In general, there were very few differences between the surface antigen phenotypes of marrow and blood leukemic cells. In five cases one or two major differences were observed (an antigen was "positive" in one source and "negative" in the other). For all comparative data in this report, bone marrow results were used when both were available, and when only blood was available for study, the results from blood were used.

Relationship of Surface Antigen Expression to Clinical Characteristics

FAB classification. The frequency of expression of each myeloid surface marker in relationship to central FAB classification is shown in Table 4.

Significant differences in antigen expression were observed among different FAB groups. Expression of Ia antigens was almost universal in monocytic (FAB M4, M5) leukemias (89–95%), uncommon in M3 (27%), and of intermediate frequency in M1 (77%), M2 (83%). Expression of several antigens correlated with FAB M4 or M5 (Ia, MY4, MY8, Mol, Mo2, PM-81, AML-2-23, and MY1, all P values < 01), while monocytic markers were particularly uncommon in FAB M3 (MY4 0/11, $P = .007$; Mo2 0/10, $P = .03$). Expression of MY4 tended to be more commonly observed in M5B (10/13 cases) than in M5A (3/9, $P = .08$). M2 cases tended to express "early granulocyte" antigens (those expressed on late myeloblasts and promyelocytes, but not on CFU-GM) such as MY8, Mol, MCS-1, to a higher extent than did M1 cases. Expression of several antigens found on normal hematopoietic progenitor cells (Table 1) did not correlate with FAB classification (MY7, MY9). The distribution of antigens in the major FAB classes is illustrated schematically in Fig 2. The antigens are displayed from left to right in the order in which they are acquired during normal myelopoiesis. Taken as a group, the FAB M1 cases tend to express "early" antigens to a greater degree than "later" myeloid antigens. Expression of later antigens such as MY8 and Mol is increasingly common in M2, M4, and M5B groups. Thus, certain "phenotypes" tend to be particularly common in each FAB group although there is considerable overlap.

Cytochemistry. The presence or absence of PX or ANAE was assessed in 164 and 170 cases, respectively.

Table 4. Frequency of Expression of Surface Antigens in Morphologic Subgroups of AML

Surface Antigen	Samples Tested*	Antigen Expression in FAB subgroups (%)									P
		M1	M2	M3	M3V	M4	M4E	M5A	M5B	M6	
		16-22	25-30	8-11	2-3	46-61	6-9	9-9	9-13	2-3	
1 Ia	161	77	83	27	67	95	89	89	92	33	†
2 MY9	161	68	67	73	67	67	67	100	77	67	
3 PM-81	124	100	88	62	0	98	100	89	100	50	‡
4 AML-2-23	124	62	76	25	0	87	100	78	100	50	‡
5 MY7	161	55	53	64	33	56	78	44	46	67	
6 MCS-1	141	28	44	11	0	38	56	44	78	33	
7 MY8	160	23	41	18	0	62	89	44	100	67	†
8 Mo1	160	19	47	9	0	70	67	78	100	33	†
9 MY4	161	18	30	0	0	48	78	33	77	0	†
10 Mo2	149	15	26	0	0	35	78	33	45	0	§
11 MY1	147	33	38	10	0	57	56	67	75	50	‖

*N = number of cases within each FAB category tested for expression of each antigen (range of values)

†$P < 0001$

‡$P < .001$

§$P < .01$

‖$P < 05$

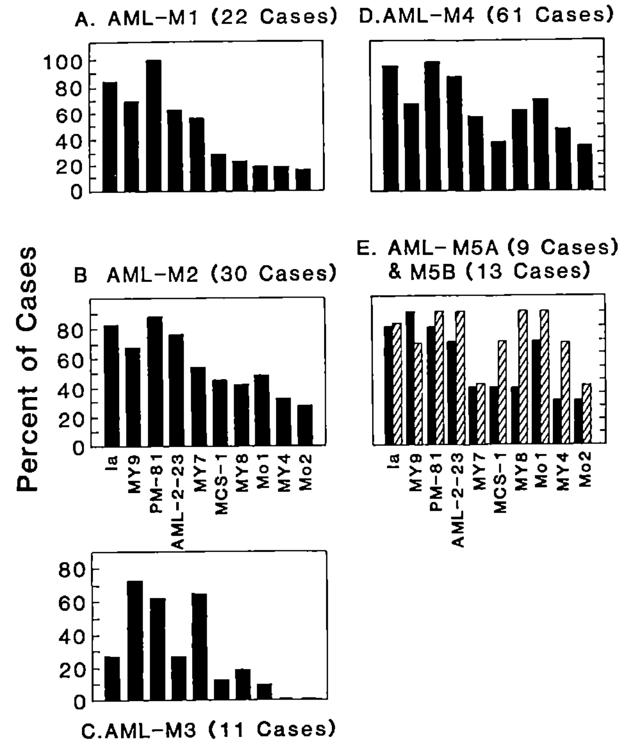

Fig 2. Antigens expressed by each FAB Group. Cases were classified by FAB criteria by the CALGB Central Review Panel, and the percent of cases in each group expressing each of the myeloid antigens is indicated. Antigens are ordered on the x axis of each panel in the order in which they are acquired during normal maturation with "early" antigens on the left and antigens of more mature cells on the right. M6 was not included because of the low number of cases. The M3 group includes only M3 cases and not M3V. In panel E, the antigens expressed by M5A cases are shown by the dark bars and the antigens expressed by M5B cases by the light bars.

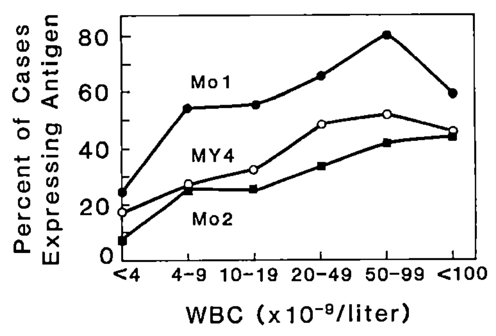

Fig 3. Relationship of expression of Mo1, MY4, and Mo2 antigens to leukocyte count. 196 cases were divided into one of six groups by white blood cell count (WBC) at presentation (x axis). The percent of cases in each group expressing Mo1, (solid circle), MY4 (open circle), or Mo2 (solid square) is indicated.

Overall, 90% of cases were PX+ and 61% were ANAE+. PX expression did not correlate with surface antigen expression. A positive correlation was detected for ANAE and expression of several individual markers, including Ia ($P = .0001$), MY8 ($P = .002$), Mol ($P = .007$), PM-81 ($P = .02$), AML-2-23 ($P = .001$) and MY1 ($P = .008$) (data not shown). The presence or absence of Auer rods did not correlate with antigen expression.

White blood cell count. The expression of three antigens (MY4, Mol, Mo2) was significantly correlated with the white blood cell count (Fig 3). The nominal P values for this association were $P = .02$ (Mo2), $P = .01$ (MY4), and $P = .0001$ (Mol).

Extramedullary disease at presentation. The presence or absence of extramedullary disease was assessed in 194 cases. Eighty-eight cases demonstrated one or more sites of extramedullary disease (Table 2). The presence of two monocyte surface antigens (MY4 and Mo2) was closely associated with the presence of any extramedullary disease. The MY4 antigen was present on the leukemic cells of 48% of 88 cases having extramedullary disease but only 28% of the 106 cases without extramedullary disease ($P = .007$). Similarly, 38% of cases with extramedullary disease were Mo2-positive compared to 22% of those without extramedullary disease ($P = .02$). Of the 11 cases with central nervous system (CNS) disease at presentation, 73% were MY4 positive while 35% without CNS leukemia expressed this antigen ($P = .02$).

Response. The relationship of antigen expression and response to therapy is shown in Table 5. Expression of two antigens was associated with low CR rates, MY4 ($P = .03$) and MY7 ($P = .01$), while expression of the other nine antigens did not predict for response to therapy. Previous CALGB studies have found patient age to be the single most important prognostic variable for induction of complete remission.[1] Neither MY7 nor MY4 expression correlated

Table 5. Relationship of Surface Antigen Expression to Response and Survival

Antigen		N	1-Year Rates (%) ± Standard Error		
			CR Rate (%)	CCR	Survival
Ia	+	160	62	50 ± 6*	43 ± 4
	−	36	67	90 ± 7	58 ± 8
MY9	+	139	65	58 ± 6	47 ± 4
	−	57	58	55 ± 12	39 ± 7
PM-81	+	129	64	52 ± 7	44 ± 5
	−	13	54	80 ± 18	54 ± 14
AML-2-23	+	109	61	47 ± 7	39 ± 5
	−	33	67	72 ± 12	68 ± 9
MY7	+	111	55*	57 ± 7	41 ± 5
	−	85	73	58 ± 8	51 ± 6
MCS-1	+	69	61	48 ± 9	38 ± 6
	−	103	60	57 ± 8	47 ± 5
MY8	+	103	59	44 ± 7*	36 ± 5*
	−	91	66	71 ± 7	55 ± 6
Mo1	+	108	61	48 ± 7*	41 ± 5
	−	85	66	72 ± 8	54 ± 6
MY4	+	72	53*	56 ± 9	41 ± 6
	−	124	69	58 ± 6	48 ± 5
Mo2	+	52	54	62 ± 11	51 ± 7
	−	126	63	52 ± 7	41 ± 5
MY1	+	81	63	51 ± 8	46 ± 6
	−	97	60	60 ± 8	47 ± 5
All cases		196	63	57 ± 5	45 ± 4

*$.01 < P <$ 05, based on the exact test for CR rates and the log-rank test for CCR and survival

Table 6. Cell Surface Phenotypes Predictive of Response

Phenotypes*		N	CR Rate	1-Year Rates (± SE) CCR	Survival
[MY4−, MY7−]	+	62	82†	61 ± 8	56 ± 7§
	−	134	54	55 ± 7	40 ± 4
[MY7−, AML-2-23−]	+	19	84§	74 ± 13	72 ± 11§
	−	162	60	52 ± 6	40 ± 4
[MY7+, MY9−]	+	27	44	61 ± 18	30 ± 10
	−	169	66	57 ± 5	48 ± 5
[MY4+, PM-81+]	+	50	44‡	56 ± 13	39 ± 8
	−	124	69	58 ± 6	48 ± 5
[MY4+, AML-2-23+]	+	49	45‡	56 ± 13	40 ± 8
	−	125	68	58 ± 6	47 ± 4
All cases		196	63	57 ± 5	45 ± 4

*"+" indicates cases with the indicated phenotype; "−" indicates all cases without the indicated phenotype

†$P <$ 001

‡$P <$ 01

§$P <$ 05

with age. To determine if MY7 and MY4 antigen expression is a poor prognostic indicator independently of age, the effects of antigen expression were analyzed after stratification into 3 age groups (<40, 40 to 59, and >60). In each age group, MY7-positive patients had a lower CR rate ($P = .02$). This was also true for MY4 expression (except in the age group 40–59 where CR rates were equal for MY4+ and MY4− patients), being most notable in those patients over 60 where 29% (8/28) of MY4+ cases achieved CR compared to 63% (22/35) of MY4− cases.

Pairwise combinations of surface antigens were also examined to assess for predictive value for high response (>80%) or low response <45%) (Table 6). The largest phenotypic group predictive for good response [MY7−, MY4−] (62 cases, CR rate 82%) was further characterized. This phenotype was not selectively expressed by any FAB group ($P = .42$), nor was there any association of this phenotype with age, sex, bleeding, infection, white blood cell count, platelet count, or treatment protocol. Specifically, the median ages of patients with the [MY7−, MY4−] phenotype and all other cases were 49 and 52.5 years, respectively. Similarly, median WBC's were 19.5 and 26.2. Thus, the [MY7−] phenotype (43% of all cases) and the [MY7− MY4−] phenotype (expressed by 32% of all cases) were associated with high CR rates without apparent association with the other known risk factors.

Remission duration and survival. The relationship between antigen expression, CCR rate after 1 year, and overall survival rate at 1 year are shown in Table 5. Expression of any of three antigens, Ia, MY8, and Mo1, was associated with decreased CCR ($P < .05$). The presence of MY8 also predicted for shorter survival ($P = .03$). CCR and survival curves for subgroups of patients defined by expression of Ia, MY4, MY7, MY8, and Mo1 are shown in Fig 4.

Expression of Lymphocyte Antigens in AML Cases

CALLA. CALLA antigen (J5 antibody) was detected on 26% of marrow leukemic cells from a 55-year-old woman with M2 leukemia. Antigen density (fluorescence intensity) was very low.

T cell markers. Samples from 3 patients with AML contained more than 20% of cells expressing the T cell marker T101. Two samples were peripheral blood, and smears in each case showed the presence of 20% to 30% lymphocytes The leukemic blasts were not felt to express the T101 antigen, but confirmatory dual fluorescence staining for coexpression of a myeloid antigen and T101 was not performed. Three other cases (all three samples were bone marrow) had more than 20% T11+ cells. Two cases were in the FAB M4E group (38% and 24% T11+ cells). In both cases T101 was detected on less than 2% of cells, and review of smears showed 10% lymphoid cells.

B cell markers. B1 was not detected in any AML cases. B4 was detected in a small number (less than 35%) of bone marrow cells in three cases. All three cases were positive for three or more myeloid markers, but negative for T cell markers, CALLA, and B1. One case was classified as M1 and two as M4.

DISCUSSION

The potential utility of surface markers in identifying patients with different prognoses has been investigated to only a limited extent, despite the fact that initial monoclonal

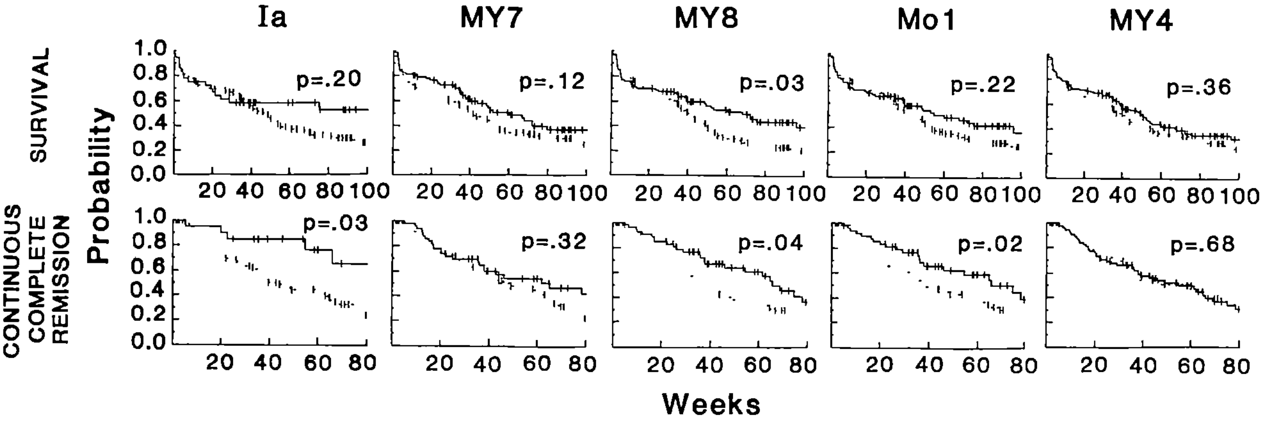

Fig 4. Kaplan-Meier survival and CCR curves for patient groups defined by expression of selected antigens (Ia, MY7, MY8, Mo1, MY4). Solid lines, antigen-negative cases; dotted lines, antigen-positive cases.

antibody studies revealed a high degree of patient heterogeneity.[27,53] The present study was undertaken in an effort to determine the clinical relevance of surface marker analysis in AML. Expression of each of 11 myeloid surface antigens was correlated with morphology and various clinical and laboratory features of 196 cases of AML (Table 2). 161 of these cases were classified using standard FAB criteria by a central panel of hematopathologists.

In this study, bone marrow aspirate cells were analyzed in 82% of cases and peripheral blood in 18%. In a pilot study of 31 cases in which both marrow and blood were available, major differences in antigen expression (defined as more than 20% difference in antigen expression, with one value positive and one value negative) were observed in five of the 31 cases. In most of these cases, major differences occurred in only one antigen of the ten tested, and this was observed randomly among the ten antigens. These results show that marrow and blood results differ in a minority of cases and for a minority of antigens examined.

As expected from previous studies with these antibodies or related antibodies,[27] expression of certain antigens correlated with FAB morphology. For example, expression of Ia, MY4, MY8, Mo1, AML-2-23, MY1, and Mo2 was significantly higher in FAB M4 and M5 than in other variants, while expression of the late monocyte markers MY4 and Mo2 was not observed in FAB M3. In the M5 group, expression of MY4 antigen was more common in M5B than in the less "differentiated" M5A. Expression of several "pan-myeloid" antigens was unrelated to FAB morphology (MY7, MY9, AML-2-23, PM-81). Antigen expression did not correlate with age, sex, the presence of myeloperoxidase, platelet count, or infection status (data not shown) The expression of monocyte markers correlated with ANAE staining and with high white blood counts, consistent with the previously reported tendency for monocytic leukemias to have extreme leukocytosis.[54] MY4 and Mo2 expression correlated with the presence of extramedullary disease, and MY4 expression was correlated with CNS leukemia in particular.

The major goal of this study was to use surface marker analysis to identify prognostically important subsets of patients. The expression of certain antigens detected on leukemic cells at diagnosis was found to correlate with subsequent CR rate, continuous complete remission duration, or survival. Expression of the MY7 antigen identified a group of patients with a lower CR rate (55%) and this was true for patients in any age group. Similarly, MY4 antigen expression was predictive of a low response rate (53%), particularly in patients over age 60 (29%). The 150-kD MY7 antigen has previously been shown to be associated with cell proliferation of normal myeloid progenitor cells [32] It is possible that the expression of higher levels of MY7 antigen in AML is associated with a more highly proliferative leukemic clone. Although the presence of both MY7 and MY4 antigens appeared to diminish the probability of achieving CR, their expression individually was not related to subsequent remission duration or survival However, longer follow-up time is necessary to determine if antigen expression predicts for prolonged disease-free survival.

Preliminary analysis of antigen combinations suggests that certain antigen "phenotypes" may be even more useful than single antigens, particularly in terms of predicting the probability of CR. For example, the [MY4− MY7−] phenotype, expressed by the cells of 62 cases (32% of the total population) had a CR rate of 82%, while the [MY4+, PM-81+] phenotype group (50 cases, 26%) had a CR rate of 44%. These results suggest that it may be possible to use a small panel of antimyeloid monoclonal antibodies to identify large subgroups of patients with dramatically different response rates to standard therapy, thereby selecting patients with very poor prognosis for whom alternative induction regimens would be appropriate.

Further prognostic information can be obtained from the surface marker analysis for patients achieving CR. The lack of expression of Ia antigen (HLA-DR) identified a group of 36 patients with a very low rate of relapse during the first year of remission (90% continuous CR) and an overall survival (all cases) of 58% after 1 year. In contrast, of 99 Ia+ cases entering CR (62% of 160 cases), only 50% remained in remission at 1 year, and overall survival was 43%. Previous studies from our laboratory and others have shown that most cases of acute promyelocytic leukemia (M3) are Ia− while only a small fraction of AML cases other than M3 lack Ia.[27] This was confirmed in the present study. Since previous studies have shown prolonged disease-free survival in M3,[17] it might be postulated that the good prognosis of Ia− cases was due to the contribution of M3 cases. However, in this study the estimated 1-year CCR rate of the ten M3 cases who entered remission was 73% (90% for the 24 Ia− cases who entered CR). Thus, Ia antigen is a useful prognostic indicator which appears to be additive to the information obtained from FAB typing.

MY8 and Mo1 antigens also provided prognostic information by identifying cases having a significantly lower continuous CR duration (44% and 48% at 1 year for MY8+ and Mo1+ cases, respectively, *v* 71% and 72% for MY8− and Mo1− cases). Overall, 1-year survival for MY8+ cases was also significantly lower than that for MY8− cases (36% *v* 55% at one year, $P < .05$) It is noteworthy that the [MY7− MY4−] phenotype (CR rate 82%) was also associated with an improvement in overall survival (56 ± 7% at 1 year *v* 40 ± 4% for all other cases, $P = .02$). Median follow-up time in this study is 19.5 months, and it will be of interest to follow these groups of cases to determine if these apparent prognostic features persist as more time elapses.

The FAB classification system in this group of patients also provided useful prognostic information (Table 3). M3 cases had a high CR rate (91% of 11 cases) and 73% of these cases with CR remained in CR at 1 year Also, although the CR rate for M4 and M5 cases was not different from the CR rate of the whole population, 1-year CCR and 1-year survival were lower, particularly in the M5A group. M4E cases tended to have a high CR rate and excellent survival, in accord with previous reports.[55,56] Thus, useful prognostic information could be abstracted from the FAB classification system. It is important to note that surface marker analyses in this study provided prognostic information independent of FAB classification. For example, the [MY7−] and [MY4− MY7−] phenotypes predictive of low CR rates were not associated with any FAB group. It is therefore possible that a maximum of prognostic information will be obtained by considering a number of clinical and biologic features of the leukemic cells in individual cases.

This study was not designed to test the accuracy of any of these monoclonal antibodies in confirming or establishing the diagnosis of AML. Most of the myeloid-reactive antibodies used here have previously been shown to have utility in distinguishing AML from ALL. It is noteworthy that only one of 196 cases (an M4) lacked expression of all myeloid antigens tested. Also, in accord with previous studies,[27] the incidence of "lymphoid" antigens in AML is very low. Although there are growing numbers of case reports of "biphenotypic" leukemias coexpressing myeloid and lymphoid characteristics,[57,58] most investigators have found this phenomenon to occur in less than 5% of acute myeloid leukemias.

The results presented here suggest that surface marker analysis in AML can be used to develop a unique classification system in AML that will provide information not obtainable by morphology or cytochemistry. The expression of many antigens is unrelated to FAB classification, and many may therefore be indicators of biologic cell functions other than differentiation. The ability to identify patient groups with poor prognosis may ultimately lead to trials of alternative chemotherapy for these patients or identify which patients should have bone marrow transplantation in first remission. Finally, as future studies identify the cell functions associated with each myeloid antigen, studies such as this are likely to provide considerable information about the derangements of gene regulation in AML.

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REFERENCES

1. Rai KR, Holland JF, Glidewell OJ, Weinberg V, Brunner K, Obrecht JP, Priesler HD, Nawabi IW, Prager D, Carey RW, Cooper MR, Haurani F, Hutchinson JL, Silver RT, Falkson G, Wiernik P, Hoagland HC, Bloomfield CD, James GW, Gottlieb A, Ramanan SV, Blom J, Nissen NI, Bauk A, Ellison RR, Kung F, Henry P, McIntyre OR, Kaan SK: Treatment of acute myelocytic leukemia: A study by Cancer and Leukemia Group B. Blood 58.1203, 1981

2. Cassileth PA, Begg CB, Bennet JM, Bozdech M, Kahn SB, Weiler C, Glictz JH: A randomized study of the efficacy of consolidation therapy in adult acute nonlymphocytic leukemia. Blood 63:843, 1984

3. Vogler WR, Winton EF, Gordon DS, Raney MR, Go B, Meyer L: A randomized comparison of postremission therapy in acute myelogenous leukemia: A Southeastern Cancer Study Group trial. Blood 63:1039, 1984

4 Mayer RJ, Schiffer CA, Peterson BA, Silver RT, Cornwell GG, McIntyre OR, Rai KR, Budman DR, Ellison RR, Maguire M, Davis RB, Frei E Intensive postremission therapy in adults with acute nonlymphocytic leukemia with Ara-C by continuous infusion or bolus administration· Preliminary results of a CALGB phase I study. Semin Oncol 12:84, 1985

5. Foon KA, Gale RP: Controversies in the therapy of acute myelogenous leukemia. Am J Med 72:963, 1982

6. Peterson BA, Bloomfield CD. Treatment of acute nonlymphocytic leukemia in elderly patients. Cancer 40:647, 1977

7. Keating MJ. Early identification of potentially cured patients with acute myelogenous leukemia—A recent challenge, in Bloomfield CD (ed). Adult Leukemias 1. Boston, Nijhoff, 1982, p 237

8. Cadman EC, Capizzi RL, Bertino JR Acute nonlymphocytic leukemia. A delayed complication of Hodgkin's disease therapy Analysis of 109 cases. Cancer 40·1280, 1977

9. Curtis JE, Messner HA, Hasselback R, Elhakim TM, McCulloch EA. Contributions of host- and disease-related attributes to the outcome of patients with acute myelogenous leukemia. J Clin Oncol 2:253, 1984

10. Spitzer G, Dicke KA, Gehan EA, Smith T, McCredie KB, Barlogie B, Freireich EJ. A simplified in vitro classification for prognosis in adult acute leukemia. The application of in vitro results in remission-predictive models Blood 48.795, 1976

11 Priesler HD. Prediction of response to chemotherapy in acute myelocytic leukemia Blood 56:361, 1980

12 Brandman J, Bukowski RM, Greenstreet R, Hewlett JS, Hoffman GC Prognostic factors affecting remission, remission duration, and survival in adult acute nonlymphocytic leukemia Cancer 44 1062, 1979

13. The Fourth International Workshop on Chromosomes in Leukemia. The clinical significance of chromosomal abnormalities in acute nonlymphoblastic leukemia Cancer Genet Cytogenet 11:332, 1984

14. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukemias. Br J Haematol 33.351, 1976

15. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C· Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

16. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposed revised criteria for the classification of acute myeloid leukemia· A report of the French-

American-British Cooperative Group Ann Intern Med 103 620, 1985

17 Bennett JM, Begg CB· Eastern Cooperative Oncology Group Study of the cytochemistry of adult acute myeloid leukemia by correlation of subtypes with response and survival Cancer Res 41:4833, 1981

18. Sultan C, Deregnaucourt J, Ko YW, Imbert M, D'Agay R, Gouault-Heilmann M, Brun B: Distribution of 250 cases of acute myeloid leukemia (AML) according to the FAB classification and response to therapy. Br J Haematol 47:545, 1981

19 Appelbaum FR, Dahlberg S, Thomas ED, Buckner CD, Cheever MA, Clift RA, Crowley J, Deeg HJ, Fefer A, Greenberg PD, Kadin M, Smith W, Stewart P, Sullivan K, Storb R, Weiden P: Bone marrow transplantation or chemotherapy after remission induction for adults with acute nonlymphoblastic leukemia. Ann Intern Med 101:581, 1984

20. Foon KA, Naiem F, Yale C, Gale RP: Acute myelogenous leukemia Morphologic classification and response to therapy. Leuk Res 3 171, 1979

21. Mertelsmann R, Thaler HT, To L, Gee TS, McKenzie S, Schauer P, Friedman A, Arlin Z, Cirrincione C, Clarkson B: Morphological classification, response to therapy, and survival in 263 adult patients with acute nonlymphoblastic leukemia. Blood 56 773, 1980

22. Nadler LM, Ritz J, Griffin JD, Todd RF, Reinherz EL, Schlossman SF: Diagnosis and treatment of human leukemias and lymphomas utilizing monoclonal antibodies, in Brown EB (ed) Progress in Hematology XII. Orlando, FL, Grune and Stratton, 1981, p 187

23. Sallen SE, Ritz J, Pesando J, Gelber R, O'Brien C, Hitchcock S, Coral F, Schlossman SF: Cell surface antigens: Prognostic implications in childhood acute lymphoblastic leukemia. Blood 55·395, 1980

24. Hoelzer D, Thiel E, Löffler H, Bodenstein H, Plaumann L, Buchner T, Urbanitz D, Koch P, Heimpel H, Engelhardt R, Müller U, Wendt F-C, Sodomann H, Rühl H, Herrmann F, Kaboth W, Dietzfelbinger H, Pralle H, Lunscken C, Hellriegel K-P, Spors S, Nowrousian RM, Fischer J, Fülle H, Mitrou PS, Pfreundschuh M, Gorg C, Emmerich B, Quiesser W, Meyer P, Labedzki L, Essers U, König H, Mainzer K, Herrmann R, Messerer D, Zwingers T: Intensified therapy in acute lymphoblastic and acute undifferentiated leukemia in adults. Blood 64·38, 1984

25. Bloomfield CD, Gajl-Peczalska KJ: The clinical relevance of lymphocyte surface markers in leukemia and lymphoma. Curr Top Hematol 3 175, 1980

26. Sobol RE, Roystan I, LeBien T, Minowada J, Anderson K, Davey FR, Cuttner J, Schiffer C, Ellison RR, Bloomfield CD· Adult acute lymphoblastic leukemia phenotypes defined by monoclonal antibodies. Blood 65·730, 1985

27. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF· Surface marker analysis of acute myeloblastic leukemia. Identification of differentiation-associated phenotypes. Blood 62 557, 1983

28 Ball ED, Fanger MW· The expression of myeloid-specific antigens on myeloid leukemia cells: correlations with leukemia subclasses and implications for normal myeloid differentiation Blood 61:456, 1983

29. Pessano S, Palumbo A, Ferrero D, Pagliardi GL, Bottero L, Lai SK, Meo P, Carter C, Hubbell H, Lange B, Rovera G Subpopulation heterogeneity in human acute myeloid leukemia determined by monoclonal antibodies. Blood 64:275, 1984

30 Van der Reijden HJ, van Rhenen DJ, Lansdorp PM, van 't Veer MB, Langenhuijsen MMAC, Englefreit CP, von dem Borne AEGK· A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443, 1983

31 Griffin JD, Ritz J, Nadler LM, Schlossman SF Expression of myeloid differentiation antigens on normal and malignant myeloid cells J Clin Invest 68.932, 1982

32. Henry JB. Clinical Diagnosis and Management by Laboratory Methods (ed 16). Philadelphia, Saunders, 1979, p 1089

33. DeSalvo Cardullo L, Morilla R, Catovsky DL: Significance of phi bodies in acute leukemia. J Clin Pathol 34:153, 1981

34 Peto R, Peto J: Asymptomatically efficient rank invariant test procedures. J Royal Sta Soc, Series A 135 185, 1972

35 Mehta CR, Patel NR: A hybrid algorithm for Fisher's exact test in unordered rxc contingency tables Commun Stat 15:387, 1986

36 Pagano M, Tritchler D: Algorithms for the analysis of several 2 × 2 contingency tables. SIAM J Sci Comput 4.302, 1983

37 Bartolucci AA. Estimation and comparison of proportions, in Buyse ME (ed). Cancer Clinical Trials: Methods and Practice. Oxford, Oxford University Press, 1984, p 337

38 Griffin JD, Ritz J, Beveridge RP, Lipton JM, Daley JF, Schlossman SF: Expression of MY7 antigen on myeloid precursor cells. Int J Cell Cloning 1:33, 1983

39. Griffin JD, Linch DC, Schlossman SF· A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8·521, 1984

40. Todd RF, Nadler LM, Schlossman SF: Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126:1435, 1981

41. Tatsumi E, Sagawa K, Mirro J, Civin CI, Preisler HD, Henderson ES, Minowada J· Immunologic membrane phenotypes in human myeloid leukemia by monoclonal antibodies. Proc Am Assn Cancer Res 22:181, 1981

42. Civin C, Mirro J, Banquerigo ML. MY1, a new myeloid specific antigen identified by a mouse monoclonal antibody Blood 57:842, 1981

43. Ball ED, Fanger MW: Monoclonal antibodies reactive with human myeloid leukemia cells Clin Exp Immunol 48:655, 1982

44. Ball ED, Kadushin JM, Schacter B, Fanger MW. Studies on the ability of monoclonal antibodies to selectively mediate complement-dependent cytotoxicity of human myelogenous leukemia blast cells. J Immunol 128:1476, 1982

45. Ball ED, Graziano RF, Fanger MW: A unique antigen expressed on myeloid cells and acute leukemia blast cells defined by a monoclonal antibody. J Immunol 130.2937, 1983

46. Todd RF, Schlossman SF. Analysis of antigenic determinants on human monocytes and macrophages. Blood 59:775, 1982

47. Todd RF, Meuer SC, Romain PL, Schlossman SF: A monoclonal antibody that blocks class II histocompatibility-related immune interactions. Hum Immunol 10:23, 1984

48. Roystan I, Majda JA, Baird SM, Meserve BL, Griffiths JC. Human T cell antigens defined by monoclonal antibodies. The 65,000-dalton antigen of T cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. J Immunol 125:725, 1980

49. Meuer SC, Hussen RE, Fabbi M, Fox D, Acuto O, Fitzgerald KA, Hodgdon JC, Protentis JP, Schlossman SF, Reinherz EL: An alternative pathway of T-cell activation: A functional role for the 50 kD T11 sheep erythrocyte receptor protein Cell 36:897, 1984

50 Stashenko P, Nadler LM, Hardy R, Schlossman SF· Characterization of a human B lymphocyte specific antigen. J Immunol 125.1678, 1980

51 Nadler LM, Anderson KC, Marti G, Bates M, Park E, Daley JF, Schlossman SF: B4, a human B lymphocyte-associated antigen expressed on normal, mitogen-activated, and malignant B lymphocytes. J Immunol 131:244, 1983

52. Ritz J, Pesando JM, Notis-McConarty J, Lazarus H,

Schlossman SF: A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature (Lond) 283:583, 1980

53. Bernstein ID, Andrews RG, Cohen SF, McMaster BE. Normal and malignant human myelocytic and monocytic cells identified by monoclonal antibodies. J Immunol 128:876, 1982

54. Cuttner J, Conjalka MS, Reilly M, Goldberg J, Reisman A, Meyer RJ, Holland JF: Association of monocytic leukemia in patients with extreme leukocytosis. Am J Med 69.555, 1980

55 Arthur DC, Bloomfield CD· Partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute nonlymphocytic leukemia. A new association. Blood 61·994, 1983

56. LeBeau MM, Larson RA, Bitter MA: Association of an inversion of chromosome 16 with abnormal marrow eosinophils in acute myelomonocytic leukemia: A unique cytogenetic-clinicopathological association. N Engl J Med 309:630, 1983

57. Bettelheim P, Paietta E, Majdic O, Gadner H, Schwarzmeier J, Knapp W. Expression of a myeloid marker on TdT-positive acute lymphocytic leukemic cells: Evidence by double-fluorescence staining Blood 60:1392, 1982

58. Neame PB, Soamboonsrup P, Browman G, Barr RD, Saeed N, Chan B, Pai M, Benger A, Wilson EC, Walker IR, McBride JA: Simultaneous or sequential expression of lymphoid and myeloid phenotypes in acute leukemia. Blood 65:142, 1985

59. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF: Leucocyte Typing. New York, Springer-Verlag, 1984

Acute Myelomonocytic Leukemia With Abnormal Eosinophils and inv(16) or t(16;16) Has a Favorable Prognosis

By Richard A. Larson, Stephanie F. Williams, Michelle M. Le Beau, Mitchell A. Bitter, James W. Vardiman, and Janet D. Rowley

Inv(16)(p13q22) and t(16;16)(p13;q22) are recurring chromosomal rearrangements which juxtapose the metallothionein gene cluster at 16q22 with other DNA sequences from 16p13. We have studied 20 men and 13 women who had acute nonlymphocytic leukemia; 27 patients had an inv(16) and six patients had a t(16;16). Eight patients also had trisomy 22, and four had trisomy 8. All but two patients had the unique morphologic features of acute myelomonocytic leukemia with abnormal eosinophils (M4Eo). In one patient with M4 leukemia, abnormal eosinophils were not observed in the marrow. A second patient had acute monocytic leukemia, plus abnormal eosinophils. Eosinophils constituted 1% to 46% (median, 6%) of the bone marrow cells, and in all but a single patient, the eosinophils exhibited distinctly abnormal morphology. Twenty-five patients have had a complete remission (78% of treated patients). Nine patients have remained in remission longer than 24 months. No patient had symptoms of central nervous system (CNS) disease at diagnosis, and none had CNS leukemic mass lesions at any time. Treatment with high-dose cytarabine may have provided prophylactic CNS therapy. Four additional patients with chromosomal rearrangements involving a breakpoint at 16q22 but not at 16p13 have had different morphological features and different clinical courses. Thus, the juxtaposition of genes at 16p13 and 16q22, which occurs both in the inv(16) and the t(16;16), results in a specific subset of acute nonlymphocytic leukemia that has a favorable prognosis.
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STRUCTURAL ABNORMALITIES of chromosome 16 have recently been identified in bone marrow cells of some patients with acute nonlymphocytic leukemia (ANLL) and have been associated with distinctive clinical, morphological, and cytochemical findings[1-3] In 1982, Arthur and Bloomfield reported on a deletion of the long arm of chromosome 16 [del(16)(q22)] in five patients with ANLL and marrow eosinophilia[3] We described a similar abnormality, a pericentric inversion of chromosome 16 [inv(16)(p13q22)], which was present in leukemia cells from 18 patients who had acute myelomonocytic leukemia (AMMoL) with morphologically and cytochemically abnormal eosinophils.[1,2] This association was confirmed by the Fourth International Workshop on Chromosomes in Leukemia,[4] and this variant of AMMoL has been designated M4Eo by the French-American-British Cooperative Group (FAB)[5] Hogge et al reported on a similar patient with AMMoL (M4Eo) and a related chromosomal abnormality, that is, a translocation between the two homologous chromosome Nos. 16, t(16;16)(p13.1;q22).[6] Other rearrangements, including a complex translocation, t(1;16;16)(q32;p13;q22), and a t(3;16)(q21;q22), have been identified by other investigators in patients who had eosinophilia and either AMMoL or a myelodysplastic syndrome.[7,8] The common features in all of these patients have been a breakpoint at 16q22 together with a malignant disease of myeloid origin and an associated qualitative or quantitative abnormality of the eosinophils.

This report details the clinical. histopathologic, and cytogenetic data on 37 patients studied at the University of Chicago. All had ANLL and a chromosomal breakpoint at 16q22. Twenty-seven patients had an inv(16); six had a t(16;16), three had a del(16)(q22), and one had a t(3;16)(q24;q22). Using these data, we have confirmed our earlier observation that patients with an inv(16) have AMMoL and qualitatively abnormal eosinophils and a favorable prognosis. Furthermore, these same features characterize the patients with a t(16;16). Of note, we have previously demonstrated that inv(16) and t(16;16) also produce similar molecular rearrangements of DNA which result in the juxtaposition of the metallothionein gene cluster at 16q22 with other DNA sequences from 16p13.[9] In contrast to the former group of patients, the four patients with either a del(16) or a t(3;16) had different clinical features and morphological findings Therefore, rearrangements of chromosome 16 with breakpoints at both 16p13 and 16q22 seem to be the specific aberrations most closely associated with M4Eo.

MATERIALS AND METHODS

Since 1970, metaphase cell preparations adequate for cytogenetic study have been obtained from bone marrow or peripheral blood cells of 226 consecutive patients with ANLL de novo who were treated at the University of Chicago[10] Marrow specimens from an additional 210 patients were referred to our cytogenetics laboratory. Each patient has been assigned a unique patient number (UPN) for this and previous publications[1,2,10] Clinical data were collected from a review of each patient's medical history and by direct communication with their referring physicians Patients were followed until death or through September 1985.

Cytogenetic analyses with quinacrine fluorescent and trypsin-

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Giemsa banding techniques were performed on bone marrow aspirates and peripheral blood cells obtained at the time of diagnosis (36 patients) or at the time of relapse (one patient).[11] We examined cells from direct preparations, from 24-hour unstimulated cultures, or from methotrexate-synchronized cells cultured for 48 hours with phytohemagglutinin-stimulated leukocyte conditioned medium Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.[12] The criteria adopted at the First International Workshop on Chromosomes in Leukemia were used for the identification of abnormal clones [13]

The diagnosis and subclassification of ANLL were based on morphological and cytochemical studies of peripheral blood smears and bone marrow aspirates and biopsy specimens obtained prior to therapy, according to the FAB cooperative group criteria in use prior to the 1985 revision.[5,14] The diagnosis of AMMoL required the presence of more than 30% blast forms in the bone marrow, with between 20% and 80% monocytic cells in the marrow and/or the peripheral blood.[14] Cytochemical reactions performed at our institution or at referring hospitals were available in most cases and included myeloperoxidase, naphthol-ASD-chloroacetate esterase, acid phosphatase, periodic acid-Schiff, and the nonspecific esterase, using alpha-naphthyl acetate, with and without sodium fluoride inhibition, and alpha-naphthyl butyrate.[2] The original slides from two referral patients (R-19 and R-22) could not be located for review.

RESULTS

Clinical findings. The clinical features of the 33 patients with M4Eo are listed in Table 1. There were no significant differences in age, sex, or initial hemogram between the 27 patients with an inv(16) and the six patients with a t(16;16). There were 20 men and 13 women. Their ages ranged from 3 to 72 years; the median age was 45 years. Eight patients were less than 30 years old. Except for one patient (UPN 2056), none of these 33 patients had received cytotoxic treatment for a previous malignant disease, and none had had a prior myelodysplastic syndrome. Patient 2056 had received radiation therapy and chemotherapy for metastic colon cancer; a therapy-related myelodysplastic syndrome (t-MDS) had been present for 3 months before acute leukemia developed.

Table 1. Clinical and Pathological Characteristics of 33 Patients with Acute Myelomonocytic Leukemia (M4Eo)

	inv(16)(p13q22)	t(16;16)(p13;q22)
Total	27	6
Men	16	4
Women	11	2
Age	43* (3-72)	52 (35-64)
Peripheral Blood		
Hemoglobin (g/dL)	9 3 (4.8-11.1)	9 3 (5 7-11 7)
Platelets ($\times 10^{-9}$/L)	47 (14-198)	48 (19-60)
White Cells ($\times 10^{-9}$/L)	34 (1.7-486 2)	32.4 (4 6-230)
Blast Forms (%)	31 (0-90)	53 (8-62)
Monocytes (%)	32 (1-79)	19 (10-50)
Eosinophils (%)	0 (0-6)	1 (0-2)
Bone Marrow		
Cellularity (%)	100 (60-100)	98 (40-100)
Blast forms (%)	49 (25-78)	54 (30-76)
Eosinophils		
Total (%)	6 (1-46)	9 (2-33)
Abnormal Granules (%)	20 (0-48)	25 (0-60)
Monocytes† (%)	29 (15-64)	32 (25-49)

Nineteen patients have been previously reported on in part [1,2]

*Median (range)

†Monoblasts, promonocytes, and monocytes

Most patients initially sought medical advice for symptoms of anemia. Sixteen patients were initially febrile or had other signs of infection. Five patients had minor bleeding. Patient R-15 had bowel obstruction by a myeloblastoma one month before AMMoL became evident in the bone marrow. Thirteen patients had lymph node enlargement, and nine had palpable splenomegaly. None of these patients had central nervous system (CNS) symptoms, but six of the sixteen patients who had a lumbar puncture performed at initial diagnosis had a few leukemia blast cells in the cerebrospinal fluid (CSF) at that time. Later, all six had normal CSF while the bone marrow was in remission. At relapse, CNS symptoms again were not noted, although three of five patients who had a lumbar puncture at that time had small numbers of blast cells in the CSF. No patient had a CNS mass lesion due to leukemia demonstrated at any time, although due to the absence of CNS symptoms computed tomography scans of the head were not commonly obtained.

Initial hemoglobin concentrations ranged from 4.8 to 11 7 gm/dL (median, 9.3 gm/dL). The initial platelet counts ranged from 14 to 198 × 10^9/L (median, 47 × 10^9/L). Initial leukocyte counts ranged from 1.7 to 486 × 10^9/L (median, 34 × 10^9/L). Eight patients had white blood cell counts greater than 100 × 10^9/L. The proportion of blasts in the peripheral blood ranged from 0% to 90% (median, 32%), and the percentage of monocytes from 1% to 79% (median, 29%). Peripheral blood eosinophilia was not prominent; eosinophils ranged from 0 to 4.9 × 10^9/L (0% to 6% of the circulating white blood cells). Half of the patients (16/33) had <1% circulating eosinophils. In all but a single patient, the eosinophils in the peripheral blood were morphologically normal. In that patient (UPN 238), only rare abnormal eosinophils were identified in the circulation. Serum muramidase (lysozyme) was elevated in the twelve patients in whom it was measured, and the urinary concentration of muramidase was increased in two additional patients.

Cytogenetic analyses. Among 436 patients with ANLL de novo and 63 patients with therapy-related ANLL who had adequate material for cytogenetic studies, 37 patients had a chromosomal rearrangement which resulted from a breakpoint in chromosome 16 at band q22. Twenty-seven patients had a pericentric inversion of chromosome 16 [inv(16)(p13q22)], whereas six patients had a similar rearrangement resulting from a reciprocal translocation involving both chromosome 16 homologues [t(16;16)(p13;q22)]. In both rearrangements, chromosomal breaks occur at bands p13 and q22, and a reciprocal exchange of chromosomal material occurs. The notable difference between these two rearrangements is that the inv(16) involves only one No. 16 homologue, whereas in the t(16;16) both chromosome 16 homologues are rearranged (Figs 1 and 2). Of the remaining four patients, three had a deletion of the long arm of chromosome 16 resulting from a break at band q22 [del(16)(q22)] and one patient (UPN 146) had complex karyotypic abnormalities which included a reciprocal translocation between chromosomes 3 and 16 [t(3;16)(q24;q22)]. The results of cytogenetic analysis of the 33 patients with M4Eo, each of whom

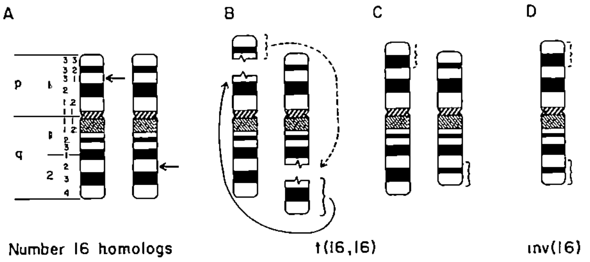

Fig 1. Schematic diagram of the banding pattern of (A) the normal chromosome 16 homologues, (B) the mechanism of the translocation 16;16, (C) the banding patterns of the rearranged chromosome 16 homologues resulting from the t(16;16), and (D) the banding pattern of the inverted chromosome 16. In (A) the arrows identify the breakpoints of the inv(16) and the t(16;16). The dashed and solid braces (C,D) identify the identical chromosomal junctions resulting from the inv(16) and the t(16;16).

had an inv(16) or a t(16;16), are listed in Table 2; sixteen of these patients were from the University of Chicago series (60 through 238, 2056, 8013) and 17 were referral patient specimens. The pretreatment karyotypes of the four patients who had a del(16) or a translocation involving 16q22 are listed in Table 3.

Among the 33 patients with M4Eo, 23 patients had a mixture of normal and abnormal mitotic cells. Eighteen patients had an inv(16) or a t(16;16) as their sole karyotypic abnormality. The remaining 15 patients had additional chromosomal abnormalities; an extra chromosome 22 was present in six of them (179, R-17, R-24, R-26, R-28, R-29). This abnormality was also observed as a nonclonal abnormality in two other patients (111, R-2) The next most frequent secondary change was a gain of chromosome 8, which was noted at initial diagnosis in four patients (176, R-1, R-25, R-26) and at relapse in two others (R-15, R-21). An interstitial deletion of the long arm of chromosome 7 was observed in three patients (182, 183, 2056), including the single patient (UPN 2056) who had received cytotoxic treatment for a previous malignant disease. The deletion involved different regions in each patient: bands q32q36, q21q31, and q31q34, respectively. With respect to these secondary changes, a gain of chromosome 8 and a loss of all or part [del(7q)] of chromosome 7 are frequently observed in the malignant cells of patients with ANLL. However, the apparently high frequency of trisomy 22 present in eight of our 33 patients is notable in that we have observed this abnormality in only five of 403 ANLL patients in the absence of an inv(16) or a t(16;16).

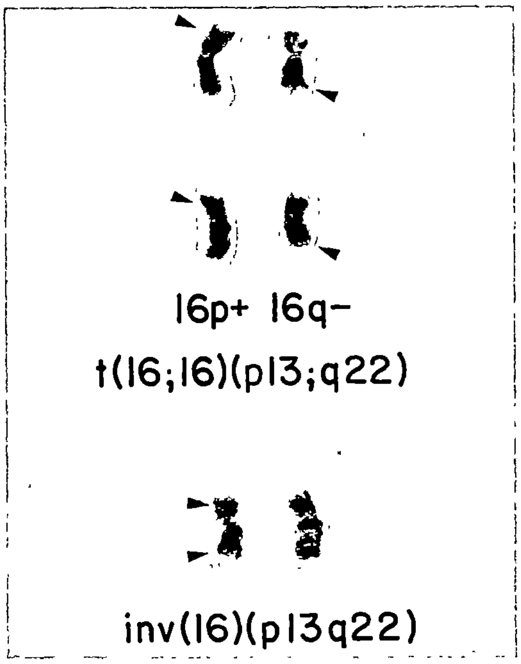

Fig 2. Partial karyotypes of trypsin-Giemsa-banded metaphase cells showing the translocation 16;16 (two metaphase cells) from Patient R-28 and the inverted chromosome 16 (one metaphase cell) from Patient R-26.

Three patients (60, 179, 2056) who were included in our earlier report on 18 patients with an inv(16) were initially interpreted to have both an inv(16) and a del(16).[1] After a review of metaphase cells from each of these patients, including the preparation of new slides, the karyotypes of all three patients were reclassified as having a t(16;16), rather than two rearranged 16 homologues arising from an inversion and a deletion.

Cytogenetic analysis of bone marrow obtained during complete remission in 16 patients (Table 2) showed a disappearance of the abnormal clone and a return to a chromosomally and histologically normal marrow. Nine patients have relapsed; one of these patients (R-26) was initially examined by our laboratory only at the time of relapse. The remaining eight patients were examined both at the time of diagnosis and at the time of relapse. Of these, four patients (168, 183, R-19, R-25) had identical karyotypes at both analyses, whereas the other four patients (176, R-15, R-21, R-28) showed karyotypic evolution at relapse. Two patients (R-15, R-21) acquired a gain of chromosome 8 in addition to the inv(16) at relapse, and Patient 176 had a t(2;?) (q33;?) at relapse that was not observed at the time of diagnosis. Finally, Patient R-28 had a t(16;16) and a +22 at diagnosis, but at relapse, she had these abnormalities as well as a t(3;9) (q23;p24), a del(15)(q13q15), and a gain of the deleted chromosome 15.

Morphologic features. The 15 patients with M4Eo de novo initially diagnosed at the University of Chicago were 21% of our 73 patients with AMMoL seen between 1970 and 1985. Patients with either an inv(16) or a t(16;16) showed similar morphologic features in the bone marrow and peripheral blood (Table 1). Furthermore, the morphology of the leukemia did not differ significantly between those patients with an additional chromosome 22 and those lacking a +22. All but two patients (R-25 and R-29) with an inv(16) or a t(16;16) had all of the typical morphological features of AMMoL with abnormal bone marrow eosinophils, characterized by the presence of eosinophils with large basophilic staining granules in Wright-stained aspirate specimens.[1,2] These eosinophil abnormalities were not observed in the 58 other patients with AMMoL seen at the University of Chicago in whom an inv(16) or a t(16;16) were not present. Patient R-29 had both AMMoL and a t(16;16), but the bone

Table 2. Cytogenetic Features of Patients with an inv(16) or a t(16:16)

Patient No	Specimen*	Method†	Number of Metaphase Cells	Percent Abnormal	Karyotype‡
Patients with an inv(16)(p13q22)§					
111‖	BM	24 hr	19	100	46,XX,inv(16)(p13q22)(94%)/NCA: 47,XX,+22,inv(16)(6%)
238¶	BM	Direct	3	0	46,XX/46,XX,inv(16)(p13q22)
		24 hr	13	54	
		48 hr	14	71	
8013¶	BM	24 hr	10	70	46,XY/46,XY,inv(16)(p13q22)
		48 hr	12	42	
R-2‖	BM	48 hr MTX	16	87	46,XY/46,XY,inv(16)(p13q22)(81%)/NCA. 46,XY,−15,+22,inv(16)(6%)
R-17	BM	24 hr	19	95	46,XY/47,XY,+22,inv(16)(p13q22)(85%)/ 46,XY,inv(16)(10%)
		48 hr MTX	1	100	
R-18	BM	24 hr	5	80	46,XY/46,XY,inv(16)(p13q22)(56%)/NCA· 47,XY,+mar(6%)
		48 hr	11	55	
R-19	BM	48 hr	8	100	46,XY,inv(16)(p13q22)
R-20¶	BM	48 hr MTX	15	93	46,XY/46,XY,inv(16)(p13q22)
R-21	BM	24 hr	9	100	46,XX/46,XX,inv(16)(p13q22)
		48 hr MTX	15	87	
R-22	BM	Direct	2	50	46,XX/46,XX,inv(16)(p13q22)
		24 hr	8	75	
	PB	48 hr MTX	9	100	
R-23	BM	Direct	1	100	46,XY/46,XY,inv(16)(p13q22)
		24 hr	11	82	
R-24	BM	24 hr	7	100	46,XY,inv(16)(p13q22)(87%)/46,XY,−17,−17,+22, inv(16),+der(17)t(17;17)(p13;q2?1)(13%)
		48 hr	9	100	
R-25	BM	24 hr	9	100	46,XX/47,XX,+8,inv(16)(p13q22)
		48 hr	8	88	
R-26¶#	BM	24 hr	18	95	46,XX/47,XX,+8,inv(16)(p13q22)(39%)/ 48,XX,+8,+22,inv(16)(56%)
Patients with a t(16,16)(p13;q22)					
60‖¶	BM	Direct	26	46	46,XX/46,XX,t(16;16)(p13;q22)
179‖¶	BM	24 hr	40	100	47,XY,+9,t(16,16)(p13,q22)(11%)/ 48,XY,+9,+22,t(16;16)(86%)/NCA: 48,XY,−8,+9,+9,+22,t(16;16)(3%)
2056‖	BM	24 hr	24	88	46,XY/46,XY,del(7)(q31q34),t(16,16)(p13;q22)
R-27	BM	24 hr	14	100	46,XY,t(16,16)(p13;q22)
		48 hr	13	100	
R-28	BM	24 hr	11	100	47,XX,+22,t(16,16)(p13;q22)(95%)/NCA· 46,XX,t(16;16)(5%)
		48 hr	11	100	
R-29¶	BM	24 hr	11	100	46,XY,t(16;16)(p13,q22)(73%)/ 47,XY,+22,t(16,16)(27%)
		48 hr MTX	11	100	

*BM, bone marrow; PB, peripheral blood

†Direct refers to direct preparations, 24 hr, cells cultured for 24 hours, 48 hr MTX, cells cultured for 48 hours in phytohemagglutinin-stimulated leukocyte conditioned medium and synchronized with methotrexate

‡NCA, nonclonal abnormality

§Karyotypes from 13 patients have been previously published and are not listed here.[1]

‖Previously published karyotype has been revised.[1]

¶Analysis of a subsequent bone marrow specimen obtained during complete remission revealed a normal karyotype.

#Initial cytogenetic analysis performed only at the time of clinical relapse.

marrow eosinophils were not increased in number and those seen were normal in appearance. Abnormal marrow eosinophils were easily identified in Patient R-25, but her leukemia was classified as M5b because 85% of the marrow cells were monocytoid. Since many cases in this series were evaluated at other institutions, cytochemical reactions and lysozyme measurements were frequently not available. However, the absolute peripheral blood monocyte count was less than 5 × 10^9/L in 10 of the 33 patients (30%) with the inv(16) or the t(16;16), and we suspect several of these patients would be reclassified as M2 if the recently revised FAB criteria were used.[5]

In contrast to patients with an inv(16) or a t(16;16), bone marrow eosinophils were neither increased in number, nor were they abnormal-appearing in the patient (UPN 146) with the 3;16 translocation or in the three patients (197, 215,

Table 3. Cytogenetic Features of Four Patients with a del(16) or a t(3;16)

Patient No	Specimen*	Method†	Number of Metaphase Cells	Percent Abnormal Cells	Karyotype‡
One Patient with a t(3;16)(q24,q22)					
146	BM	48 hr MTX	8	100	45,XY,−3,−12,−16,−17,del(5)(q23q33),+der(12) t(3;12)(p14,p13),+der(16)t(3;16)(q24;q22),+mar[del(3) (p12q11)](21%)/44,XY,same,−18,t(11;?)(p15,?)(68%)/ NCA:46,XY,same clone 1,+21(5%)/44,XY,same clone 2, t(X;14)(q23;q32)(5%)
	PB	24 hr	11	100	
Three Patients with a del(16)(q22)					
197	BM	Direct	2	0	46,XY/46,XY,del(16)(q22)(11%)
		24 hr	24	13	
		48 hr MTX	2	0	
215	BM	24 hr	6	0	46,XY/46,XY,del(16)(q22)(9%)
	PB	Direct	2	0	
		24 hr	25	12	
R-30¶	BM	24 hr	15	53	46,XY/46,XY,del(16)(q22)(47%)/NCA.47,XY,+mar(6%)

See Table 2 footnotes.

R-30) with a del(16) (Table 4) When first seen, Patient 146 had refractory anemia with excess blasts in transformation. His disease subsequently progressed to frank acute leukemia which was classified as AMMoL. Patient 197 had a previous diagnosis of chronic myelomonocytic leukemia (CMMoL), which evolved into AMMoL after one year. The initial bone marrow biopsy from Patient 215 performed at another institution showed CMMoL. Upon admission to the University of Chicago, bone marrow necrosis was observed. Additional biopsies obtained after the initiation of therapy showed acute leukemia. The bone marrow from Patient R-30 showed a leukemic process in which the promonocyte was the predominant cell type. His leukemia was classified as M5b. It is of interest that three of the four patients with a del(16) or the 3;16 translocation had evidence of a myelodysplastic process prior to the onset of acute leukemia. In addition, each of these four patients had a leukemic process with monocytic participation.

Response to treatment. Thirty-two patients with an inv(16) or a t(16;16) received intensive remission induction chemotherapy using cytarabine infusion with or without an anthracycline drug plus other agents. Twenty-five patients (78%) achieved a complete remission (CR), and most of these patients were in remission after the initial treatment course. In one patient (R-23), CR was achieved only after allogeneic bone marrow transplantation following several unsuccessful attempts with conventional chemotherapy. Patient 2056 with therapy-related acute leukemia (t-ANLL) achieved CR after a single six-day course of high-dose cytarabine (HDAC). Two patients died within 2 weeks of diagnosis and treatment, and five patients died during the initial period of marrow hypoplasia (3 from sepsis [*S aureus, E coli, Candida*], one from intracranial hemorrhage, and one from renal and hepatic failure). One patient (R-1) declined to receive chemotherapy and died of pneumonia 2 weeks after diagnosis.

The median duration of remission for all 25 patients will be longer than 65 weeks as 15 patients remain in first CR (Fig 3). Two patients have died in remission. Patient 111 died from intracranial hemorrhage while thrombocytopenic following consolidation chemotherapy. Patient 2056 died of preexistent metastatic colon carcinoma. Eight patients have relapsed with a median duration of first remission of 63 weeks (range, 35 to 91 weeks). All but one have achieved a second remission, and five of these patients are still living. Patient R-15 had a second remission of 6 months, and Patient 168 had a second remission of 8 months, both eventually died with progressive leukemia. Patient R-21 died of complications following bone marrow transplantation.

Most patients received additional postremission chemo-

Table 4. Clinical and Pathological Characteristics of Four Additional Patients with a del(16)(q22) or a t(3;16)

			Peripheral Blood						Bone Marrow				
											Eosinophils		
Patient No	Age/Sex	Diagnosis	Hemoglobin g/dL	Platelets $\times 10^{-9}$/L	White Cells $\times 10^{-9}$/L	Blast forms %	Monocytes %	Eosinophils %	Cellularity %	Blast forms %	Total %	Abnormal Granules %	Monocytic Cells %
One patient with a t(3,16)(q24,q22)													
146	72/M	MDS → M4	9.3	59	5.5	48	4	1	90	33	5	0	2
Three patients with a del(16)(q22)													
197	78/M	CMMoL → M4	10.6	117	75 2	1	40	1	100	22	1	0	50
215	63/M	CMMoL → M4	11 0	70	33 1	2	40	0	100	<30	0	0	4
R-30	19/M	M5b	7.8	71	249	3	NA	0	NA	39	0	0	80

Abbreviations· MDS, myelodysplastic syndrome, CMMoL, chronic myelomonocytic leukemia; M4, acute myelomonocytic leukemia; M5b, acute well-differentiated monocytic leukemia, NA, not available. Monocytic cells include monoblasts, promonocytes, and monocytes

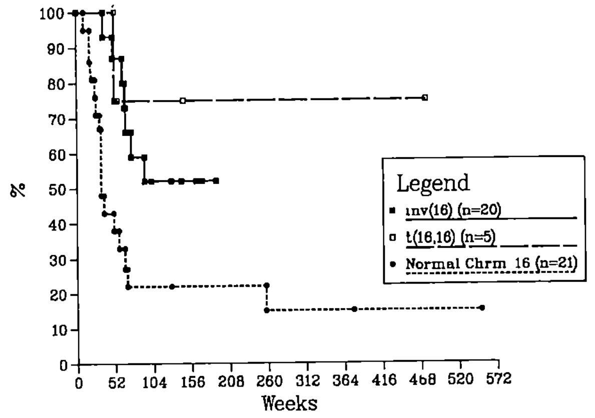

Fig 3. Curves showing the duration of first remission for patients with an inv(16) or a t(16;16) compared to all other patients with AMMoL (M4) treated at the University of Chicago. The duration of remission was significantly longer in patients with these specific abnormalities of chromosome 16 than in all other M4 patients ($P = 0.02$).

therapy. Eight patients received at least two courses of high-dose cytarabine (2 to 3 gm/m^2) for consolidation, and only one of these patients (UPN 183) has relapsed. This latter patient was put back into a durable CR with a single course of HDAC. Of the eight patients who have had a relapse, six were treated at that time with HDAC, and all six regained CR. One patient regained CR with a cytarabine-anthracycline-thioguanine regimen. Of interest, two patients have had unmaintained second CRs lasting longer than 80 weeks after receiving only a single course of HDAC for reinduction therapy and then no further therapy.

The median survival for all 32 treated patients is longer than 66 weeks, and the median survival for those 25 patients who had a CR is longer than 104 weeks (Fig 4). Nine patients have been in unmaintained remission longer than 2 years after diagnosis and may be free of residual disease, since the latest observed relapse has been at 91 weeks.

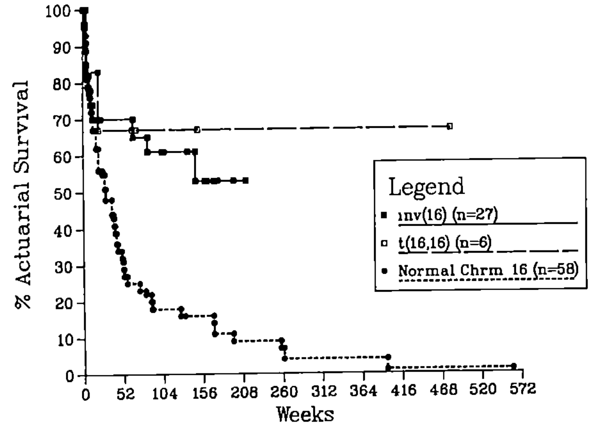

Fig 4. Overall survival curves for all 33 patients with an inv(16) or a t(16;16) compared to all other patients with AMMoL (M4) treated at the University of Chicago. There was no significant difference in survival between the inv(16) and the t(16;16) groups. However, the survival of these two groups was significantly better than that of all other M4 patients ($P = 0.01$).

During this same period (1970–1985), AMMoL de novo was diagnosed in 58 other patients at the University of Chicago. None of these patients had abnormal eosinophils, nor did they have any abnormalities of chromosome 16. Using similar chemotherapy regimens, 21 patients (36%) achieved a CR, but only five (9%) are currently living. The median duration of first remission was 33 weeks, and the median survival for the whole group was 29 weeks. Only four patients were still in remission after 2 years, and one of these relapsed after five years.

Other patients with a breakpoint at 16q22. We have studied four other patients, not included in the above analyses, in whom bone marrow metaphase cells had a breakpoint at 16q22. Their clinical and morphologic findings are listed in Table 4, and their karyotypes are listed in Table 3. All four were men. Three had a del(16)(q22) but did not have a rearrangement of 16p. Two of these patients (197, 215) were older men (78 and 63 years old, respectively) who had had a primary myelodysplastic syndrome (chronic myelomonocytic leukemia) for several months before the development of AMMoL. The third patient with a del(16)(q22) was young (19 years old) and had well-differentiated acute monocytic leukemia (M5b) at initial presentation. The fourth man was 72 years old and had also had a myelodysplastic syndrome prior to developing AMMoL. His bone marrow karyotype had a t(3;16)(q24;q22) and a del(5)(q23q33) among other abnormalities. The short arms (16p) of both chromosomes 16 were normal. None of these four patients had morphologically or cytochemically abnormal eosinophils, nor were their eosinophils increased in number in peripheral blood or marrow. The youngest patient achieved a CR with conventional cytarabine and anthracycline chemotherapy. One year later he had a relapse, but he regained CR with HDAC. Of the three elderly patients, one (146) was not treated, and the other two received low-dose cytarabine therapy (20 mg/m^2/day) but failed to respond. One of the latter two patients (215) subsequently also failed to respond to HDAC therapy.

DISCUSSION

We have reported on 27 patients with an identical pericentric inversion of chromosome 16 and six additional patients with a translocation involving both chromosome 16 homologues. This translocation is notable because it involves the same breakpoints on the long and short arms of the chromosome 16 homologues as does the inversion and, therefore, results in a similar juxtaposition of DNA.[9] All but two patients had the identical morphological and cytochemical features of AMMoL with abnormal eosinophils (M4Eo). In one of these, abnormal eosinophils could not be identified, and in the other, the leukemia was classified as M5b due to the presence of 85% monocytoid cells in the marrow. As a group, these patients have had a good response to intensive chemotherapy programs with no discernible differences between the two cytogenetic forms.

As mentioned above, we have subclassified these patients morphologically, using the FAB criteria for AMMoL published in 1976.[14] Using this classification, all but one patient (UPN R-25, who had 85% monoblasts, promonocytes, and monocytes in the marrow aspirate) clearly met the criteria for AMMoL, ie, between 20% and 80% monocytoid cells in the marrow and/or blood. The most recently proposed revised criteria for M4 from the FAB Group may not accommodate all such cases with an inv(16) or a t(16;16), since several of our cases would have been classified as M2 (acute myeloblastic leukemia with maturation) using the later criteria.[5] Nevertheless, the eosinophil abnormality and the clear-cut evidence of monocytic involvement still provide morphologic homogeneity for this subgroup of leukemia patients with a consistent chromosomal abnormality. A similar situation exists with acute promyelocytic leukemia in which the presence of a t(15;17)(q22;q21) accurately identifies every case, including both the hypergranular and the microgranular variants.[15]

In support of our hypothesis that the juxtaposition of DNA from both 16q22 and 16p13 is required for this distinct phenotype, we have presented data on four additional patients with different clinical and morphological features. Each of these patients also had a breakpoint at 16q22, but 16p13 was not involved in any case. In contrast to the patients with an inv(16) or a t(16;16), all of whom presented with ANLL de novo (except one who had t-ANLL), three of the four additional patients had a primary myelodysplastic syndrome which later transformed to AMMoL. These three individuals were elderly men and were not candidates for intensive chemotherapy. Of note, in the recent series reported from the M.D. Anderson Hospital, the single patient (their #23) with a del(16q) was a 78-year-old woman with a prior myelodysplastic syndrome.[16] None of our four additional patients had the abnormal eosinophils with an admixture of large basophilic granules characteristic of the inv(16) and the t(16;16), nor were the marrow eosinophils increased in number as has been reported by other investigators who have described patients with ANLL and del(16)(q22).[3]

Whether recognized by its distinctive morphology or by its two consistent cytogenetic abnormalities, AMMoL with abnormal eosinophils identifies a group of patients with a good prognosis Seventy-eight percent of the M4Eo patients described in this paper have achieved a complete remission following induction chemotherapy. Following intensive consolidation regimens using chemotherapy or marrow transplantation, remissions appear to be durable, and nine patients have remained in remission for more than 2 years after diagnosis. Both remission duration and survival were significantly longer for these M4Eo patients than for the 58 other AMMoL patients who did not have abnormalities of chromosome 16 and were treated during the same time period (Figs 3 and 4). Of the 67 patients with M4Eo reported to date in the literature, 54 (81%) have entered complete remission, and 38 (57%) were still alive at the time of reporting.[1,2,6,7,16]

Complications of CNS leukemia were not frequent among our patients in direct contrast to a recent report from the M.D. Anderson Hospital.[16] Six of 25 patients (24%) reported on by Holmes et al developed intracerebral myeloblastomas. None of our 33 patients have had such a finding. Six of our 16 patients who had a lumbar puncture performed at initial diagnosis had a few leukemia blast cells in the CSF, although none had neurological symptoms. In every case, the CSF was free of leukemia following systemic induction chemotherapy by the time the marrow was in remission. Specific intrathecal therapy was not employed Many of our patients received one or more courses of high-dose cytarabine after remission, and since this regimen produces cytotoxic drug levels within the CSF, this may be directly responsible for our low rate of CNS leukemic relapse.

Clonal abnormalities can now be detected cytogenetically in most, if not in all, patients with ANLL, and more subtle clonal abnormalities are no doubt present in other patients at the molecular level. With improvements in chemotherapy and supportive therapy, the presence or absence of a detectable cytogenetic abnormality is no longer in itself predictive of the achievement of a complete remission. However, at the Fourth Workshop, prognostic significance for remission duration, and thus survival, appeared to reside in specific and recurring abnormalities.[4] For example, patients with a t(15;17) or a t(8;21), had a longer survival than did those with a del(5q) or a loss of No. 5. As evidence accumulates that patients with a "favorable" malignant clone may have extended survival and perhaps cure with intensive but conventional chemotherapy programs, it may become reasonable to exclude such good prognosis patients from more experimental therapies, such as allogeneic marrow transplantation, during first remission.

These unique chromosomal rearrangements and the clinical entity described in this report assume added importance because of recent results of genetic analysis of translocation breakpoints and because of our increasing understanding of cellular transforming genes. We have previously shown that the breakpoint at 16q22 splits the metallothionein gene cluster.[9] This family of low molecular weight proteins functions to regulate trace metal metabolism and to protect against heavy metal toxicity. Myeloid cells, particularly eosinophils and other granulocytes and monocytes, have high concentrations of zinc and zinc metalloenzymes. Other metal-ion binding and transporting proteins, such as transferrin and transferrin receptor, also seem to have a regulatory role in the proliferation of certain cells. Pertinent to this discussion is our recent observation that the rearrangements of chromosome 3, inv(3) and t(3;3), seen in some patients with ANLL and thrombocytosis brings into close proximity the transferrin gene at 3q21 with the transferrin receptor gene at 3q26.[17,18]

Exactly how rearrangements of DNA from both 16p13 and 16q22 are associated with the clonal expansion of malignant myeloid cells awaits a better understanding of the genes present at these breakpoints. Metallothioneins may have an important role in granulocyte and monocyte differentiation. It is noteworthy that the specific morphologic abnormality associated with this leukemia is the accumulation of eosinophils with immature and dysplastic granules in the bone marrow. Alternatively, a metallothionein gene may play a different role, one in which its transcriptional control elements are involved in the activation of an as yet unidentified cellular gene located on 16p13 whose function is analogous to that of a cellular oncogene. Future work may answer these questions. For the present, it is important to recognize that patients with the M4Eo phenotype may have a favorable prognosis with currently available therapies.

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REFERENCES

1. Le Beau MM, Larson RA, Bitter MA, Vardiman JW, Golomb HM, Rowley JD: Association of an inversion of chromosome 16 with abnormal marrow eosinophils in acute myelomonocytic leukemia. A unique cytogenetic-clinicopathological association. N Engl J Med 309:630, 1983

2. Bitter MA, Le Beau MM, Larson RA, Rosner MC, Golomb HM, Rowley JD, Vardiman JW: A morphologic and cytochemical study of acute myelomonocytic leukemia with abnormal marrow eosinophils associated with inv(16)(p13q22). Am J Clin Pathol 81:733, 1984

3. Arthur DC, Bloomfield CD: Partial deletion of the long arm of chromosome 16 and bone marrow eosinophilia in acute nonlymphocytic leukemia. A new association. Blood 61:994, 1983

4. Fourth International Workshop on Chromosomes in Leukemia, 1982: Correlation of morphology and karyotype. Cancer Genet Cytogenet 11.275, 1984

5. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposed revised criteria for the classification of acute myeloid leukemia: A report of the French-American-British Cooperative Group. Ann Intern Med 103:626, 1985

6. Hogge DE, Misawa S, Parsa NZ, Pollak A, Testa JR: Abnormalities of chromosome 16 in association with acute myelomonocytic leukemia and dysplastic bone marrow eosinophils. J Clin Oncol 6·550, 1984

7. de la Chapelle A, Lahtinen R: Chromsome 16 and bone marrow eosinophilia. N Engl J Med 309:1394, 1983

8. Neri G, Daniel A, Hammond N: Chromosome 16, eosinophilia, and leukemia. Cancer Genet Cytogenet 14:371, 1985

9. Le Beau MM, Diaz MO, Karin M, Rowley JD: Metallothionein gene cluster is split by chromosome 16 rearrangements in myelomonocytic leukaemia. Nature 313·709, 1985

10. Larson RA, Le Beau MM, Vardiman JW, Testa JR, Golomb HM, Rowley JD: The predictive value of initial cytogenetic studies in 148 adults with acute nonlymphocytic leukemia: A 12 year study (1970–1982). Cancer Genet Cytogenet 11:219, 1983

11. Le Beau MM: Cytogenetic analysis of hematologic malignancies, in Wahrenburg G (ed)· Cytogenetics Laboratory Manual. Association of Cytogenetic Technologists, 1984

12. Harnden DG, Klinger HP (eds): ISCN (1985): An International System for Human Cytogenetic Nomenclature. Basel, Karger, 1985, p 1

13. First International Workshop on Chromosomes in Leukaemia: Chromosomes in acute non-lymphocytic leukaemia. Br J Haematol 39:311, 1978

14. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976

15. Larson RA, Kondo K, Vardiman JW, Butler AE, Golomb HM, Rowley JD: Evidence for a 15;17 translocation in every patient with acute promyelocytic leukemia. Am J Med 76.827, 1984

16. Holmes R, Keating MJ, Cork A, Broach Y, Trujillo J, Dalton WT Jr, McCredie KB, Freireich EJ: A unique pattern of central nervous system leukemia in acute myelomonocytic leukemia associated with inv(16)(p13q22). Blood 65.1071, 1985

17. Bitter MA, Neilly ME, Le Beau MM, Pearson MG, Rowley JD: Rearrangements of chromosome No. 3 involving bands 3q21 and 3q26 are associated with normal or elevated platelet counts in acute nonlymphocytic leukemia. Blood 66:1362, 1985

18. Le Beau MM, Diaz MO, Yang F, Plowman GD, Brown JP, Rowley JD: Molecular analysis of transferrin, transferrin receptor, and p97 antigen genes in the inv(3) and t(3;3) in acute nonlymphocytic leukemia. Am J Hum Genet A31, 1985 (abstr. 085)

Extracellular Deposition of Eosinophil Granule Major Basic Protein in Lymph Nodes of Patients With Hodgkin's Disease

By J.H. Butterfield, G.M. Kephart, P.M. Banks, and G.J. Gleich

Lymph nodes from each of the four histologic types of Hodgkin's disease were examined for the presence of eosinophils and for eosinophil degranulation by immunofluorescent localization of eosinophil granule major basic protein (MBP). Eosinophil degranulation shown by MBP deposition outside of eosinophils was found in six of eight nodes from patients with nodular sclerosing disease and in two of eight nodes from patients with lymphocyte depletion–type disease. Three nodes of the mixed cellularity type, one node of the lymphocyte predominance type, and one lymph node of the lymphocyte depletion type showed one or two small foci of extracellular MBP deposition. Lymph nodes from patients without Hodgkin's disease showed no extracellular deposition of MBP. Large numbers of eosinophils were found in seven of eight nodes of the nodular sclerosing variant, but were less frequently seen among the other types of Hodgkin's disease. The presence of extracellular MBP in lymph nodes of patients with Hodgkin's disease indicates that eosinophil degranulation commonly occurs and suggests that the released eosinophil granule proteins may participate in the inflammatory reaction in this disorder more extensively than is presently appreciated.

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EOSINOPHIL INFILTRATION into lymph nodes of patients with Hodgkin's disease was first noted nearly 100 years ago.[1,2] A review published in 1902 described lymph node eosinophilic infiltration in three fourths of the cases of Hodgkin's disease and noted that the lymph node involvement by eosinophils occurred without a parallel rise in circulating eosinophil numbers.[3] It has been suggested that eosinophils may be part of the inflammatory response to Hodgkin's disease.[4] Evidence for this hypothesis has been provided by the finding that supernatants of lymph node cultures from five of six cases of Hodgkin's disease were relatively more chemotactic for peripheral blood eosinophils than for neutrophils. In contrast, two supernatants from lymph node cultures of lymphocytic lymphoma and from four nodes showing reactive hyperplasia were chemotactic predominantly for neutrophils.[5]

Because of our interest in the function of eosinophils and eosinophil proteins, we examined lymph nodes from patients with Hodgkin's disease for evidence of eosinophil granule major basic protein (MBP) deposition. We found large deposits of extracellular MBP in six of eight nodes having nodular sclerosing histology and two of eight nodes of lymphocyte depletion histology.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded lymph node tissue specimens were obtained from the tissue registry of the Mayo Clinic Six-micron–thick sections were cut and the tissue applied to glass microscope slides with LePage's Fast Bond glue Immunofluorescent staining for MBP was performed as described previously using a two-step procedure employing affinity-purified antibody to MBP; protein A–purified normal rabbit IgG was used as the negative control.[6-8] Hematoxylin-eosin (HE)-stained slides were examined for histologic confirmation of Hodgkin's disease classification.

The slides were examined and photographed using a Zeiss microscope (Carl Zeiss, Oberkochen, FRG) equipped with both standard light and fluorescence systems After photographing the immunofluorescence findings, certain of the sections stained with anti-MBP were submerged in phosphate-buffered saline to remove the coverslip and restained with HE for histologic examination of the fluorescent areas.

To test the possibility that tissue manipulation caused eosinophil degranulation and MBP release, a lymph node was obtained at the time of biopsy from an additional, ninth, patient with nodular sclerosing Hodgkin's disease. The lymph node was cut in half, and one piece was intentionally crushed several times by finger pressure. The two halves of the node were then fixed, embedded, and stained in identical fashion

RESULTS

The results are shown in Table 1 and Figs 1 and 2. Table 1 indicates that the nodular sclerosing type showed marked eosinophil infiltration and striking eosinophil degranulation. The Figures show that three general patterns of staining were evident: (1) infiltration by intact eosinophils with no extracellular deposition of MBP, (2) areas showing both intact eosinophils and extracellular staining for MBP, and (3) areas showing extracellular deposition of MBP without the presence of any intact eosinophils. In a given lymph node one or more of these patterns could be observed (Figs 1 and 2). Of interest is the finding of extracellular MBP deposition in a "moth-eaten" pattern (Fig 2C); careful inspection of the MBP deposition showed that it outlined apparently intact cells within the tissue.

Lymph nodes from patients having the nodular sclerosing type of Hodgkin's disease showed large numbers of eosinophils as well as areas of extracellular MBP. Charcot-Leyden crystals, additionally, were noted in two nodular sclerosing lymph nodes The other types of Hodgkin's disease, especially the mixed-cellularity type, occasionally showed eosinophils scattered throughout the lymph node. However, areas of extracellular MBP were rare, and when found, were small and localized save for two lymph nodes of the lymphocyte depletion type. These two nodes also showed large areas of

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Table 1. Summary of Staining Patterns

Type of Hodgkin's Disease	Sex	Age	Diagnosis or Stage	Eosinophils in Peripheral Blood (%)	Chemotherapy Before Biopsy?	Radiation at Biopsy Site Before Biospy?	Biopsy		Staining	
							Site	Date	Eosinophils*	Extracellular Deposits
Nodular sclerosis										
Patient 1	M	23	IIIB	1	yes	yes	SC	1984	1+	rare
Patient 2	F	41	IIIA	0	no	no	neck	1984	3+	large patches
Patient 3	M	37	IIA	NA	no	no	SC	1984	2+	fine granular deposits
Patient 4	M	27	IIA	0	no	no	SC	1984	3+	small areas at edges of node
Patient 5	M	28	IIA	9–13	yes	yes	axilla	1979	3+	large bright areas
Patient 6	F	33	IIIA	0	yes	yes	neck	1979	3+	bright areas
Patient 7	F	39	IIA	NA	no	no	neck	1984	3+	large bright areas
Patient 8	M	27	IV	3	no	no	neck	1984	3+	large patches
Lymphocyte predominance										
Patient 1	M	60	IA	2	no	no	neck	1982	3+	no
Patient 2	M	77	IIIA	0.2	no	no	neck	1983	1+	no
Patient 3	M	25	IIA	0	no	no	neck	1982	0	no
Patient 4	M	29	IA	2	no	no	SC	1979	1+	small area
Patient 5	F	21	IIIA	5	no	no	inguinal	1984	0	no
Patient 6	M	50	NA (recurrence)	0.9	no	yes	axilla	1979	0	no
Patient 7	M	10	IA	2	no	no	neck	1979	1+	no
Patient 8	F	31	NA (recurrence)	0	no	yes	neck	1980	1+	no
Mixed cellularity										
Patient 1	F	63	NA	NA	no	no	neck	1984	3+	no
Patient 2	F	40	IIA	5	no	no	neck	1983	2+	no
Patient 3	F	33	I or IIB	5	yes	yes	axilla	1976	3+	few small foci
Patient 4	M	75	IVB	2	no	no	neck	1983	1+	no
Patient 5	M	20	III or IVB	1	no	no	axilla	1983	1+	no
Patient 6	F	18	III	4–13	no	no	scalene	1979	1+	few small foci
Patient 7	M	80	IA	0	no	no	neck	1984	1+	no
Patient 8	M	21	IA	NA	no	no	neck	1982	3+	few small foci
Lymphocyte depletion										
Patient 1	M	83	IIIB	0	no	no	neck	1981	1+	few small foci
Patient 2	M	15	IIIA	1	no	no	neck	1980	0	no
Patient 3	M	69	IV	2	no	no	neck	1980	1+	no
Patient 4	M	37	IIA	2–5	no	no	neck	1980	1+	no
Patient 5	F	69	IVB	0–1	no	no	SC	1980	3+	diffuse granular deposits
Patient 6	F	74	IIA	NA	no	no	parotid	1983	0	no
Patient 7	M	24	IIA	1	yes	yes	axilla	1975	0	no
Patient 8	M	14	IIIB	1	no	no	SC	1980	3+	large areas
Controls										
Patient 1	NA	NA	normal	NA	no	no	neck	1984	0	no
Patient 2	M	19	Burkitt's	3	no	no	ileum	1980	0	no
Patient 3	M	62	Burkitt's	0–1	no	no	axilla	1979	0	no
Patient 4	M	33	B cell lymphoma	4–7	no	no	axilla	1975	1+	no
Patient 5	M	13	idiopathic follicular hyperplasia	0–1	no	no	axilla	1977	1+	no

Table 1. continued

Type of Hodgkin's Disease	Sex	Age	Diagnosis or Stage	Eosinophils in Peripheral Blood (%)	Chemotherapy Before Biopsy?	Radiation at Biopsy Site Before Biospy?	Biopsy		Staining	
							Site	Date	Eosinophils*	Extracellular Deposits
Patient 6	F	28	follicular, small cleaved cell lymphoma	1	no	no	SC	1982	1+	no
Patient 7	F	52	mastectomy	NA	no	no	axilla	1981	1+	no
Patient 8	M	38	sarcoid	0–1	no	no	SC	1984	0	no
Patient 9	F	45	mastectomy	NA	no	no	axilla	1984	2+	no
Patient 10	F	48	mastectomy	NA	no	no	axilla	1984	2+	no
Patient 11	F	56	mastectomy	0	no	no	axilla	1985	1+	no

Abbreviations SC, supraclavicular, NA, not available or not determined

*0, no eosinophils, 1+, few eosinophils; 2+, eosinophils conspicuous, 3+, dense eosinophil infiltration

MBP deposition. All lymph node sections incubated with protein A–purified rabbit IgG were negative. HE restaining of lymph nodes with extracellular MBP deposition showed that these areas often contained cell debris and/or degenerating cells with pyknotic nuclei. It is noteworthy that the degree of eosinophil infiltration or presence of extracellular MBP was not related to the peripheral blood eosinophil count (Table 1).

Eleven slides consisting of one normal lymph node as well as lymph nodes from two cases of Burkitt's lymphoma, one case of diffuse immunoblastic lymphoma of B cell phenotype, one case of follicular small cleaved cell lymphoma, one case of sarcoidosis, four cases of hyperplastic normal axillary lymph node tissue from mastectomy cases, and one case of idiopathic follicular hyperplasia revealed no extracellular MBP staining even though eosinophils were present (Table 1).

Examination of the traumatized and untraumatized halves of the nodular sclerosing node failed to reveal any difference in extracellular staining by MBP. In addition, intact eosinophils were numerous in both the crushed and noncrushed halves.

DISCUSSION

Few studies have dealt with eosinophil involvement in lymph nodes of patients with Hodgkin's disease. The degree of eosinophil infiltration may be variable,[3,9] and foci of eosinophils, neutrophils, and/or plasma cells in lymph nodes may be an early indication of Hodgkin's disease.[10] The presence of unique "small-type cytoplasmic granules" in the mature eosinophils infiltrating human lymph nodes and spleen involved by Hodgkin's disease has been reported The small granules differed from and often outnumbered typical eosinophil crystalloids and were absent from early eosinophil forms in the bone marrow.[11] In a review of 327 biopsies of lymph nodes from patients with Hodgkin's disease, eosinophil infiltration amounting to more than 50% of the cell population was found in ten cases.[12] Among these ten cases, four patients had the nodular sclerosing type, four had the lymphocyte depletion type, one had features of both nodular sclerosing and mixed cellularity, and one had nodular sclerosing in association with lymphocyte depletion. Patients having lymph node eosinophil infiltration experienced significantly shorter survivals than did controls. In another series 45 lymph node specimens from 38 patients with Hodgkin's disease and five cervical lymph nodes from patients with reactive follicular hyperplasia were examined. No significant difference was found between the percentages of eosinophils in reactive follicular hyperplasia nodes (0%) and the percentages of eosinophils in lymphocyte predominance nodes ($\bar{x} = 0.25\%$); however, significant increases in the percentages of eosinophils were found in nodular sclerosing nodes ($\bar{x} = 13.2\%$, $P < .02$) and lymphocyte depletion nodes ($\bar{x} = 22.6\%$, $P < .001$).[13] The presence of eosinophils in lymph nodes of young patients proved to be the feature with the most discriminating power in separating younger-age (15 to 34 years) patients with Hodgkin's disease from the cohort of Hodgkin's patients over the age of 50 years.[14] Increased numbers of eosinophils and Reed-Sternberg cells and a high score for the extent of lymph node fibrosis were all features of nodular sclerosing Hodgkin's disease.[15]

Here we report that lymph nodes with nodular sclerosing morphology showed the heaviest infiltration with eosinophils and the most pronounced extracellular deposits of MBP. Although lymph nodes from other histologic classes did show eosinophils, extracellular MBP deposition was limited to small areas except in two lymph nodes from patients with the lymphocyte depletion type. Extracellular MBP deposition was not due to artifactually induced eosinophil degranulation because a lymph node intentionally traumatized prior to fixation, embedding, and staining did not show an increase in MBP deposition. Although the node was purposely compressed repeatedly by finger pressure, numerous intact eosin-

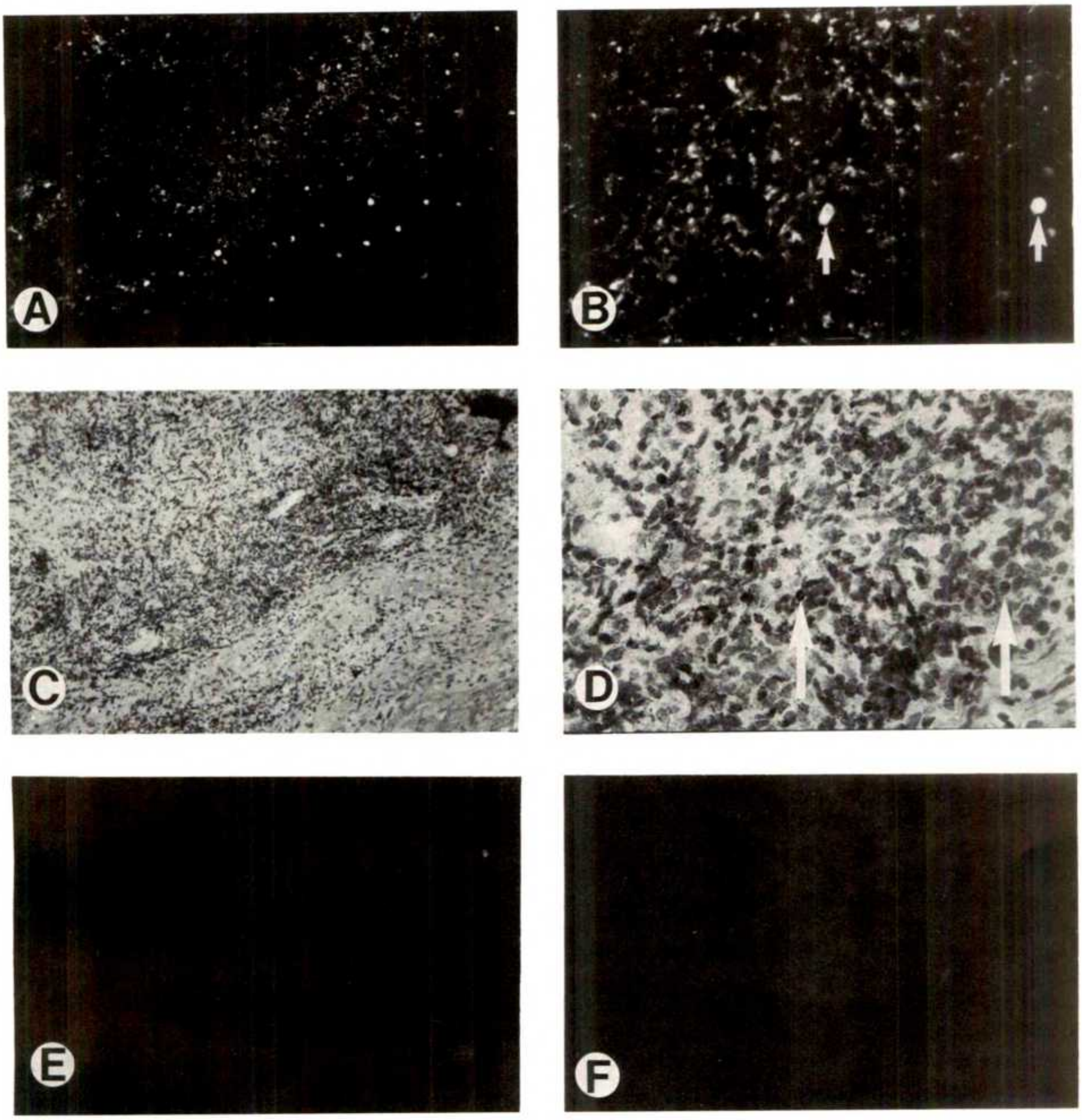

Fig 1. Lymph node from a patient with nodular sclerosing Hodgkin's disease. (A) Immunofluorescent stain of an area showing the presence of intact eosinophils as well as extracellular deposition of MBP. Original magnification × 100. (B) Enlargement of (A). Intact eosinophils are indicated by arrows. Extracellular MBP can be seen surrounding and outlining other cells in the node. Original magnification × 400. (C) HE counterstain of the identical tissue specimen present in (A) showing the inflammatory cellular infiltrate present. Intact eosinophils are not readily apparent. Original magnification × 100. (D) HE counterstain of (B) showing round cell infiltrate present in the region having extracellular MBP deposition. Intact eosinophils are indicated by arrows. Original magnification × 400. (E) Negative control. Serial section of identical tissue to (A) stained with normal rabbit IgG and showing the absence of nonspecific staining. Original magnification × 100. (F) Negative control. Serial section of identical tissue as is shown in (B) stained with normal rabbit IgG and showing the absence of nonspecific staining. Original magnification × 400.

ophils were present. This indicates that mechanical factors were not responsible for release of MBP.

As the functions of eosinophils have been elucidated, a better appreciation of this cell's role in the immune system has evolved. MBP, a low-molecular–weight protein forming the core of the eosinophil granule,[16] is toxic to murine tumor cells, guinea pig spleen cells, human mononuclear cells, helminths,[17–20] and human tracheal epithelial cells.[21] MBP is found in high concentrations in the sputum of patients with bronchial asthma and in the blood of patients with eosinophilia.[22,23] Although it is not known whether MBP is toxic to Hodgkin's disease lymphocytes, eosinophils are capable of killing antibody-coated tumor cells,[24] and MBP is toxic to human mononuclear leukocytes.[20] We have not previously

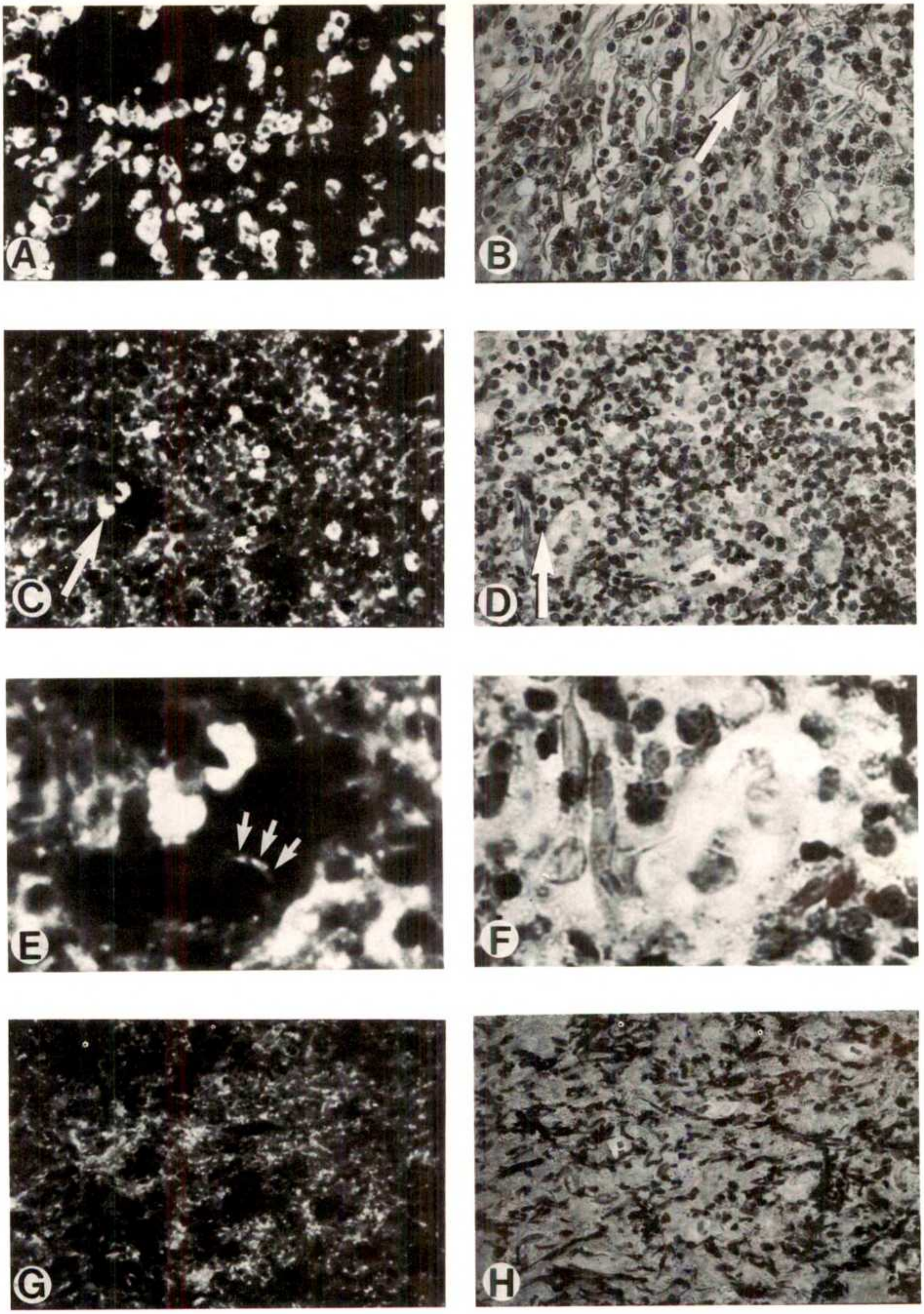

Fig 2. Sections taken from a second patient with nodular sclerosing Hodgkin's disease show the three patterns of staining noted. All original magnifications × 400. (A) Immunofluorescent staining with anti-MBP demonstrates numerous intact eosinophils with minimal extracellular staining. (B) HE restain of (A) confirms the presence of numerous eosinophils (arrow). (C) Immunofluorescent staining of a different region of same lymph node shows few intact eosinophils (arrow). In contrast, considerable extracellular MBP deposition yielded a moth-eaten appearance. (D) HE restain of (C) demonstrating intact eosinophil (arrow) but giving no indication of the presence of extracellular MBP deposition. (E) Enlargement of (C) showing two intact eosinophils as well as granular deposition of MBP around the margin of a cell (small arrows). Other cells outlined by MBP appear as dark holes on a background of bright immunofluorescence. (F) Enlargement of (D) showing the location of the intact eosinophils as well as numerous mononuclear cells that are surrounded by MBP on (E). There is no indication of the striking MBP deposition on this HE-stained slide. (G) Immunofluorescent staining demonstrates an area of extracellular MBP in a region lacking intact eosinophils. (H) HE restain of (G) showing the presence of cells with pyknotic nuclei, nuclear remnants, and debris. No intact eosinophils are seen.

observed the moth-eaten appearance of tissue shown in Fig 2C. This appearance is evidently caused by MBP deposition around cells in the tissue outlining the margins of the cells. Because MBP is toxic to lymphocytes, this pattern of deposition suggests that these labeled cells may be under attack by eosinophils through MBP. One could hypothesize a chain of inflammatory events proceeding in such lymph nodes beginning with infiltration by eosinophils (Figs 2A and 2B), followed by degranulation of eosinophils with deposition of granule products on the surface of cells (Figs 2C through 2F), and terminating with cell death occurring in a "sea" of MBP in the absence of intact eosinophils (Figs 2G and 2H). The presence of extracellular MBP in Hodgkin's disease lymph nodes signals a more common and extensive participation of eosinophils in the cellular infiltrate of these nodes than would be appreciated by standard staining methods and suggests that eosinophils and their products cause cell damage in Hodgkin's disease.

The reason why eosinophil degranulation was not invariably associated with eosinophil infiltration is obscure, but the discrepancy could reflect the influence of eosinophil-activating factors derived from mast cells, monocytes, and T lymphocytes.[25-27] These factors, alone or in combination, could determine whether the eosinophil degranulates. Also, our studies provide a view of eosinophil degranulation at a single time point, and the possibility exists that sequential studies might show the sequence of events, seen in Fig 2, of eosinophil infiltration, followed by eosinophil degranulation, and culminating in apparent cell necrosis. In addition, although we have localized MBP in this study and have stressed its potential role in the presumed cell death, as shown by Figs 2G and 2H, we recognize that other eosinophil proteins are also likely released, including the eosinophil peroxidase that in conjunction with H_2O_2 and halide is toxic to tumor cells[28,29] and eosinophil-derived neurotoxin and eosinophil cationic protein, which are toxic to cerebellar Purkinje cells. Finally, the control lymph nodes from patients undergoing mastectomy, as well as the nodes from patients with the mixed cellularity and lymphocyte predominance forms of Hodgkin's disease, while demonstrating eosinophil infiltration, do not show evidence (or at most, slight evidence) of eosinophil degranulation. Thus, nodes from patients with the nodular sclerosis form of Hodgkin's disease differ from the others in ways that we do not presently understand.

REFERENCES

1. Dreschfeld J: Clinical lecture on acute Hodgkin's disease. Br Med J 1:893, 1892
2. Reinbach I: Uber das Verhalten der Leukozyten bei maligen Tumoren. Arch Klin Chir 46:486, 1893
3. Reed DM: On the pathological changes in Hodgkin's disease, with especial reference to its relation to tuberculosis. Johns Hopkins Hosp Rep 10:133, 1902
4. Stuart AE: Immunological aspects of reticulum cell neoplasia. Br Med J 4:423, 1970
5. Kay AB, McVie JG, Stuart AE, Krajewski A, Turnbull LW: Eosinophil chemotaxis of supernatants from cultured Hodgkin's lymph node cells. J Clin Pathol 28:502, 1975
6. Filley WV, Ackerman SJ, Gleich GJ: An immunofluorescent method for specific staining of eosinophil granule major basic protein. J Immunol Methods 47:227, 1981
7. Filley WV, Holley KE, Kephart GM, Gleich GJ: Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. Lancet 2:11, 1982
8. Peters MS, Schroeter AL, Kephart GM, Gleich GJ: Localization of eosinophil granule major basic protein in chronic urticaria. J Invest Dermatol 81:39, 1983
9. Slaughter DP, Economou SG, Southwick HW: Surgical management of Hodgkin's disease. Ann Surg 198:705, 1958
10. Strum SB, Rappaport H: Significance of focal involvement of lymph nodes for the diagnosis and staging of Hodgkin's disease. Cancer 25:1314, 1970
11. Parmley RT, Spicer SS: Cytochemical and ultrastructural identification of a small type granule in human late eosinophils. Lab Invest 30:557, 1974
12. Toth J, Dworak O, Sugar J: Eosinophil predominance in Hodgkin's Disease. Z Krebsforsch 89:107, 1977
13. Fuggle WJ, Crocker J, Smith PJ: A quantitative study of eosinophil polymorphs in Hodgkin's disease. J Clin Pathol 37:267, 1984
14. Newell GR, Cole SR, Miettinen OS, MacMahon B: Age differences in the histology of Hodgkin's disease. J Natl Cancer Inst 45:311, 1970
15. Lukes RJ, Butler JJ: The pathology and nomenclature of Hodgkin's disease. Cancer Res 26:1063, 1966
16. Gleich GJ, Loegering DA, Frigas E, Wassom DL, Solley GO, Mann KG: The major basic protein of the eosinophil granule, physiochemical properties, localization and function, in Mahmoud AAF, Austen KF (eds): The Eosinophil in Health and Disease, Orlando, FL, Grune & Stratton, 1980, p 79
17. Butterworth AE, Wassom DL, Gleich GJ, Loegering DA, David JR: Damage to schistosomula of *Schistosoma mansoni* induced directly by eosinophil major basic protein. J Immunol 122:221, 1979
18. Wassom DL, Gleich GJ: Damage to *Trichinella spiralis* newborn larvae by eosinophil major basic protein. Am J Trop Med Hyg 28:860, 1979
19. Kierszenbaum F, Ackerman SJ, Gleich GJ: Destruction of bloodstream forms of *Trypanosoma cruzi* by eosinophil granule major basic protein. Am J Trop Med Hyg 30:775, 1981
20. Gleich GJ, Frigas E, Loegering DA, Wassom DL, Steinmuller D: Cytotoxic properties of the eosinophil major basic protein. J Immunol 123:2925, 1979
21. Frigas E, Loegering DA, Gleich GJ: Cytotoxic effects of guinea pig eosinophil major basic protein on tracheal epithelium. Lab Invest 42:35, 1980
22. Frigas E, Loegering DA, Solley GO, Farrow GM, Gleich GJ: Elevated levels of the eosinophil granule major basic protein in the sputum of patients with bronchial asthma. Mayo Clin Proc 56:345, 1981
23. Ackerman SJ, Loegering DA, Gleich GJ: The human eosinophil Charcot-Leyden crystal protein: Biochemical characteristics and measurement by radioimmunoassay. J Immunol 125:2118, 1980
24. Sanderson CJ, Thomas JA: A comparison of the cytotoxic activity of eosinophils and other cells by ^{51}Chromium release and time lapse microcinematography. Immunology 34:771, 1978
25. Thorne KJI, Richardson BA, Veith MC, Tai P-C, Spry CJF, Butterworth AE: Partial purification and biological properties of an eosinophil-activity factor. Eur J Immunol 15:1083, 1985

26. Warren DJ, Sanderson CJ: Production of a T-cell hybrid producing a lymphokine stimulating eosinophil differentiation. Immunology 54:615, 1985

27. Vadas MA, Nicola NA, Lopez AF, Metcalf D, Johnson D, Pereira A: Mononuclear cell–mediated enhancement of granulocyte function in man. J Immunol 133:202, 1984

28. Jong EC, Klebanoff SJ: Eosinophil-mediated mammalian tumor cell cytotoxicity: Role of the peroxidase system. J Immunol 124:1949, 1980

29. Henderson WR, Chi EY, Jong EC, Klebanoff SJ: Mast cell–mediated tumor-cell cytotoxicity. J Exp Med 153:520, 1981

The Influence of Purified Recombinant Human Heavy-Subunit and Light-Subunit Ferritins on Colony Formation In Vitro by Granulocyte-Macrophage and Erythroid Progenitor Cells

By Hal E. Broxmeyer, Li Lu, David C. Bicknell, Douglas E. Williams, Scott Cooper, Sonia Levi,
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Purified recombinant human heavy subunit (rHF, acidic) and recombinant human light subunit (rLF, basic) ferritins were assessed for their effects in vitro on colony formation by normal human granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitor cells. The purity of the samples was confirmed by electrophoresis in both nondenaturing and denaturing conditions and silver staining. Concentrations of 10^{-8} to 10^{-10} mol/L rHF caused an ~40% significant decrease in colony formation. Some significant activity was detected at 10^{-11} mol/L, and activity was lost at 10^{-12} mol/L. In contrast, rLF had no significant activity at 10^{-8} to 10^{-16} mol/L. rHF was significantly active against mouse bone marrow CFU-GM to concentrations as low as 10^{-8} to 10^{-9} mol/L. The inhibitory activity of rHF was inactivated with three different monoclonal antibodies recognizing the heavy subunit of ferritin, but not with two monoclonal antibodies recognizing the light subunit of ferritin. The inhibitory activity of rHF was similar in the absence or presence of serum, monocytes, and T lymphocytes. We and others have shown an association of a glycosylated natural acidic isoferritin (AIF) with inhibitory activity, but since the rHF was expressed in *Escherichia coli* and did not bind to concanavalin A, glycosylation of AIF is not an absolute prerequisite for this activity. These results demonstrate that rHF has suppressive activity in vitro and substantiate our original observations using purified natural acidic isoferritins.

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FERRITINS are metal-binding proteins with 24 subunits.[1,2] Isotypes of ferritin exist and are composed of varying proportions of heavy (H) subunits, with molecular weights of approximately 21,000, and light (L) subunits, with molecular weights of approximately 19,000. The more acidic isoferritins (AIF) are composed of a greater proportion of H to L subunits, and the more basic isoferritins are composed of a greater proportion of L to H subunits.[3,4]

We reported a role for AIFs as suppressor molecules of in vitro myeloid colony formation from granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and multipotential progenitor cells based on the use of purified preparations of natural AIF and the capacity of both polyclonal and monoclonal antibodies, which recognize AIFs, to inactivate the inhibitory effects of AIFs.[5-9] Others have substantiated the suppressive effects of AIF by using our preparation of chronic myelogenous leukemia–spleen AIF,[10,11] but our proposed role for AIF has been questioned by one group.[12] We have commented on their discussion of the characteristics of AIF and our noted inhibitory activity,[13] and on their data and interpretations.[14] Recently our results have been supported by data from several different groups of investigators.[15-17]

It was our belief that definitive proof for the suppressive activity of AIF in vitro would require the use of purified preparations of recombinant ferritins. The complementary (c) and genomic DNAs coding for ferritin H- and L-subunits have been cloned and sequenced.[18-24] The cDNAs coding for the two subunits have also been cloned in vectors for overproduction in *Escherichia coli*.[23,25] The two peptides are expressed with high efficiency and readily assemble into full ferritin molecules that have been characterized.[22,23] This offered us the opportunity to determine whether the recombinant H- or L-subunit ferritins had suppressive activity. Our results reported here demonstrate that purified recombinant human H-subunit ferritin (rHF), but not recombinant L-subunit ferritin (rLF), inhibits colony formation by bone marrow CFU-GM and BFU-E in vitro in the presence or absence of serum, monocytes, and T lymphocytes.

MATERIALS AND METHODS

Cells and cell separation procedures. Bone marrow cells were obtained from normal healthy donors who had given informed consent. The cells were separated by Ficoll-Hypaque (density, 1.077 g/cm³; Pharmacia Fine Chemicals, Piscataway, NJ) into a low-density population. Nonadherent low-density cells were obtained after adherence of cells to plastic tissue culture dishes (35 mm, Corning Glass Works, Corning, NY) in culture medium containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT) for 1.5 hours at 37 °C. Nonadherent cells were collected by gently swirling the dishes and slowly pipetting off the culture medium. Nonadherent low-density cells were treated with neuraminidase-treated sheep erythrocytes to remove T lymphocytes.[26] The purity of blood cells types was determined either by staining with Wright-Giemsa and nonspecific esterase or by the monoclonal antibodies OKT3 and OKT11a (Ortho Diagnostic Systems, Inc, Raritan, NJ), which recognize T lymphocytes, by using immunofluorescence with a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Sunnyvale, CA) or by direct fluorescent microscopy.[26]

Mice (BDF_1 and DBA/2) were purchased from Cumberland

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Farms Inc, Clinton, TN. Mouse bone marrow nucleated cells were used without density cut or adherence separation.

Preparation of ferritins and antiferritin monoclonal antibodies. Human heart ferritin was a kind gift from Dr. Marianne Dorner, University of Heidelberg. Liver ferritin and nonglycosylated human heart ferritin was purified as described.[15] rHF was obtained from the *E coli* clone pEMBLex2HFT, which contains a plasmid that directs the synthesis of the complete coding region of the cDNA of the H subunit of human ferritin.[23] The recombinant ferritin was purified as described in Levi et al[23]: induced cells were sonicated and the extract heated at 75 °C and the soluble proteins passed on a Sepharose 6B column and then centrifuged for 90 minutes at 200,000 g. Further purification was achieved by immunochromatography on monoclonal antiheart ferritin 2A4-Sepharose (Pharmacia, Piscataway, NJ). The recombinant ferritin obtained had structural and immunochemical properties similar to natural heart ferritin and was composed of the H subunit.[23]

Recombinant L-chain ferritin was obtained from the clone pEXHF14,[25] which contains a plasmid that directs the synthesis of the coding region of the L chain of human ferritin. Briefly, the cDNA insert of the L ferritin clone p6808 was inserted into a derivative of the expression vector pPlc24[27] constructed in order to express foreign genes without fusions. For the construction pEXHF14, a restriction site overlapping with the AUG (adenine, uracil, guanine) initiation codon was used. As a consequence, the amino acid positions 2 and 3 are changed from Ser-Ser to Asp-Pro. These exchanges at the very end of the protein are not expected to have any structural consequence. This notion is supported by Arosio et al (unpublished observations) and is in agreement with the known overall structure of the ferritin shell in which the very *N* terminus of the monomers has no function in the polymerization process. This recombinant protein was extracted from the induced cells by sonication and purified by heat treatment at 75 °C, gel filtration on Sephacryl S-200, and ion exchange chromatography on a diethyl aminoethyl column. Further purification was achieved by immunochromatography on monoclonal antiliver ferritin–Sepharose. This ferritin has structural and immunologic properties similar to natural liver ferritin (Arosio, unpublished observations).

All the monoclonal antibodies used here have been described.[8,22,28-30] The 2A4, H103, and H104 antibodies were elicited by human heart ferritin and are specific for the H chain. L03 and L04 antibodies were elicited by human liver ferritin and are specific for the L-subunit.

Analysis of ferritins with sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Ferritin samples were set up in sample buffer (0.1 mol/L Tris, pH 6.8, containing 10% glycerol and 2% SDS, Biorad Laboratories, Richmond, CA) and analyzed under nondenaturing conditions on 5% gels.[31] For denaturing conditions, preparations were set up in sample buffer with the addition of 0.1 mol/L DL-dithiothreitol (Sigma, St Louis) and boiled for three minutes before analysis on linear gradient gels of 5% to 15% acrylamide.[32]

Colony assays for hematopoietic progenitor cells. CFU-GM colony (>50 cells/aggregate) formation of human and mouse bone marrow cells were stimulated respectively by 10% exogenously supplied granulocyte-macrophage colony-stimulating factors (GM-CSF) present in medium conditioned by the urinary bladder carcinoma cell line 5637 or by pokeweed mitogen–stimulated mouse spleen cell–conditioned medium (CM).[6] These sources of CM were used because they are free of other known inhibitory molecules such as AIF and E-type prostaglandins, and when used with prescreened heat-inactivated FBS (56 °C for 30 minutes), give maximal colony formation and increase the sensitivity of bone marrow cell to inhibition by AIF compared with other sources of GM-CSF such as human placental CM and WEHI-3 CM.[6] Cells were plated in 1 mL of culture medium containing 0.3% agar (Difco Laboratories, Detroit), McCoy's 5A medium supplemented with essential and nonessential amino acids, glutamine, serum, asparagine, and sodium pyruvate (GIBCO Laboratories, Grand Island, NY), and 10% heat-inactivated FBS. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 at lowered (5%) O_2 tension. Low oxygen was maintained using an oxyreducer (Reming Bioinstruments, Syracuse, NY). Low O_2 tension used as suppression with AIF is as good as or better than that noted when cells are grown at usual incubation (~21%) O_2 tension. Colonies and clusters were scored after five and seven days of incubation respectively for mouse and human cells.

The BFU-E colony assay has been described.[7,33] Bone marrow cells were plated in a 1-mL mixture of Iscove's modified Dulbecco's medium (GIBCO), 0.8% methylcellulose, 30% FBS, 5% medium conditioned by 5637 cells, 5×10^{-5} mol/L 2-mercaptoethanol, 0.5 units of erythropoietin (Toyoba–New York, Inc, New York), and 1×10^{-4} mol/L hemin (Eastman Kodak, Rochester, NY). Hemin increases colony formation by BFU-E.[33] Dishes were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 5% O_2 and colonies scored after 14 days of incubation. CFU-GM and BFU-E cultures were also set up in the absence of serum with 10 mg/mL bovine serum albumin, 300 μg/mL iron-saturated transferrin, 7.8 μg/mL cholesterol, and 280 μg/mL calcium chloride. For serum-free cultures the 5637 CM was prepared in the absence of serum, and one unit of recombinant erythopoietin (Amgen Biologicals, Thousand Oaks, CA) was used. The samples of rHF and rLF were added in combination with the agar or methylcellulose at the start of the culture.

Statistical analysis. Three to four plates were scored per point, and the probability of significant differences between samples was determined by use of Student's *t* test (two-tailed values).

RESULTS

Ferritin samples. The preparations of purified rHF and rLF used in these studies, along with two preparations of natural heart ferritin (containing H and L subunits) and a preparation of natural liver ferritin (containing mainly L subunits), are shown in Fig 1 by silver staining of samples after running them on nondenaturing PAGE (Fig 1A) and under denaturing conditions (Fig 1B). The nondenatured and denatured purified rHF are shown respectively in lane 4 of Fig 1A and lane 5 of Fig 1B. This preparation had been affinity purified using monoclonal antibody 2A4, which recognizes the H subunit. For comparison, the nondenatured and denatured preparations of impure rHF are shown respectively in lane 5 of Fig 1A and in lane 8 of Fig 1B. The nondenatured and denatured preparations of affinity-purified rLF are shown respectively in lane 2 of Fig 1A and in lane 4 of Fig 1B. After denaturation (Fig 1B), the purified rHF migrated with the H subunit of heart ferritin, and the purified rLF migrated with the L subunit of heart and liver ferritin. The extra bands seen above the ferritin preparations in Fig 1A are most likely due to aggregation of ferritin molecules since these extra bands from the different ferritin preparations have approximately the same molecular weights and after denaturation (Fig 1B) were resolved into H- or L-subunits (see especially lanes 3 to 6, Fig 1B). The area from the stacking gel to a molecular weight of approximately 60,000 is not shown for the denaturating gels in Fig 1B, but no bands were detected for lanes 2 to 7 in this area.

Influence of rHF and rLF on colony and cluster formation by CFU-GM and BFU-E in vitro. The purified rHF and

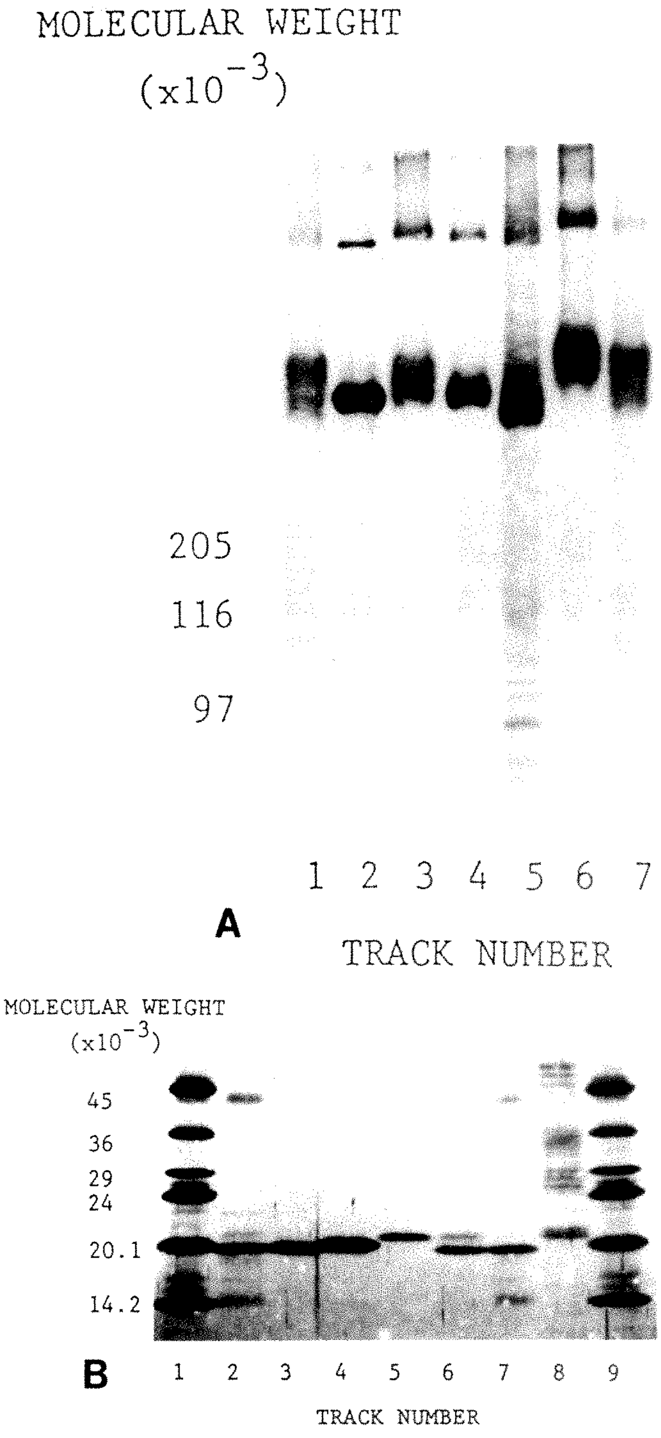

Fig 1. SDS-PAGE analysis under nondenaturing and denaturing conditions of recombinant and natural ferritins. (A) A 5% SDS-PAGE was run under nondenaturing conditions and silver stained. Tracks: 1 and 7, purified natural heart ferritin; 2, purified rLF; 3, purified natural heart ferritin from a preparation different from that shown in tracks 1 and 7; 4, purified rHF; 5, nonpurified rHF; and 6, purified natural liver ferritin. (B) The same samples were run on a 5% to 15% linear gradient SDS-PAGE under denaturing conditions and silver stained. Tracks: 1 and 9, low-molecular-weight markers; 2 and 7, purified natural heart ferritin; 3, purified natural liver ferritin; 4, purified rLF; 5, purified rHF; 6, purified heart ferritin (same sample as track 3 in part A); and 8, nonpurified rHF. The stacking portion of the gels in parts A and B are not shown.

rLF were assessed for their capacity to influence colony and cluster formation by day 7 human bone marrow CFU-GM stimulated with 5637 CM (Table 1). A plateau curve of significant inhibition of colony and cluster formation was apparent with 10^{-8} to 10^{-10} mol/L rHF. Some significant activity was still apparent at 10^{-11} mol/L, and no significant activity was apparent from 10^{-12} to 10^{-16} mol/L. Data for 10^{-14} to 10^{-16} mol/L rHF are not shown. In comparison, rLF was without effect on colony and cluster formation at concentrations of 10^{-8} to 10^{-16} mol/L. Data for 10^{-11} to 10^{-16} mol/L rLF are not shown. The suppressive activity of rHF was inactivated by preincubation of rHF with monoclonal antibodies 2A4, H103, and H104, which recognize the H subunit, but was not inactivated by preincubation of rHF with monoclonal antibodies L08 and L03, which recognize the L-subunit and immune precipitate rLF (Table 1). The unpurified preparation of rHF also significantly suppressed colony formation ($P < .005$) by 30% to 45% at concentrations of 10^{-8} to 10^{-9} mol/L in three experiments, and these effects were completely inactivated by preincubation of the

Table 1. The Influence of Purified rHF and Purified rLF on Colony and Cluster Formation by Day 7 Human and Day 5 Mouse Bone Marrow Granulocyte-Macrophage Progenitor Cells

	Percent Change From Control Medium ($\bar{Y}$ ± 1 SEM)			
	Human		Mouse	
Materials	Colonies	Colonies and Clusters	Colonies	Colonies and Clusters
rHF (10^{-7} mol/L)	ND	ND	−41 ± 2* (5)	−39 ± 3* (5)
rHF (10^{-8} mol/L)	−40 ± 3* (3)	−36 ± 3* (5)	−40 ± 5* (5)	−38 ± 4* (5)
rHF (10^{-9} mol/L)	−38 ± 2* (3)	−36 ± 3* (6)	−22 ± 2† (5)	−20 ± 3† (5)
rHF (10^{-10} mol/L)	−40 ± 2* (3)	−32 ± 3* (6)	−4 ± 2 (5)	−4 ± 3 (5)
rHF (10^{-11} mol/L)	−20 ± 5† (5)	−20 ± 4† (6)	−2 ± 2 (5)	−3 ± 1 (5)
rHF (10^{-12} mol/L)	−8 ± 6 (5)	−8 ± 5 (5)		
rHF (10^{-13} mol/L)	−8 ± 5 (5)	−6 ± 5 (4)		
rHF (10^{-14} mol/L)	−4 ± 2 (3)	−5 ± 2 (5)		
rLF (10^{-8} mol/L)	−3 ± 4 (3)	−5 ± 1 (4)		
rLF (10^{-9} mol/L)	−1 ± 3 (3)	−2 ± 2 (4)		
rLF (10^{-10} mol/L)	+2 ± 3 (3)	+3 ± 3 (4)		
rHF (10^{-9} mol/L) + MoAb2A4	−5 ± 3 (3)	−6 ± 2 (5)	−0.2 ± 4 (5)	−2 ± 2 (5)
rHF (10^{-9} mol/L) + MoAb H103	−5 ± 2 (2)	−2 ± 1 (3)		
rHF (10^{-9} mol/L) + MoAb H104	−7 ± 3 (2)	−3 ± 4 (3)		
rHF (10^{-9} mol/L) + MoAb L08	−45 ± 3* (2)	−41 ± 4* (4)		
rHF (10^{-9} mol/L) + MoAb L03	−39 ± 3* (2)	−39 ± 5* (3)		

Control (McCoy's) medium or ferritins were preincubated for 1½ hours at room temperature with control medium or 1:50 final dilutions of MoAbs 2A4, H103, or H104 or 1:20 to 1:50 final dilutions of MoAbs L08 or L03 prior to assessing for an influence on colony formation by 10^5 low-density human or 5 × 10^4 mouse bone marrow cells. For mouse cells three experiments were done with BDF_1 cells and two experiments with DBA/2 cells. Control numbers of human colonies ranged from 40 to 168, and control numbers of human colonies plus clusters ranged from 167 to 546 between experiments. Control numbers of mouse colonies ranged from 52 to 106, and control number of mouse colonies plus clusters ranged from 112 to 154 between experiments. Antibodies by themselves had no effect on human or mouse colony or cluster formation (+4% ± 5% change from control for three experiments each). Not all points were set up in each experiment, and in some experiments colonies did not form in control plates. The numbers in parentheses designate the number of experiments for each point.

Abbreviations: MoAb, monoclonal antibody; ND, not determined.

*Significant percent change from control, $P < .005$.

†Significant percent change from control, $P < .05$.

unpurified rHF with monoclonal antibody 2A4 ($P > .05$). The unpurified preparation of rHF was 1 log unit less active than the purified rHF (data not shown).

Since eventual studies of the action of these preparations in vivo would require assessing them in animal models, we evaluated the effects of rHF on colony formation by mouse CFU-GM. As seen in Table 1, rHF suppressed colony formation by mouse bone marrow cells, but the rHF was 2 log units less active than it was against human bone marrow. The suppressive effect of rHF against mouse bone marrow was inactivated with monoclonal antibody 2A4 (Table 1).

Influence of rHF and rLF on colony formation by CFU-GM and BFU-E in vitro after depletion of monocytes and T lymphocytes and in the absence of serum. The rHF, but not the rLF, also significantly suppressed colony formation by human BFU-E stimulated with erythropoietin, hemin, and 5637 CM (Table 2, one of three reproducible experiments). The suppressive effects of rHF were similar whether the target population of human bone marrow cells contained monocytes and T lymphocytes (low-density cells) or were depleted of these cells with <2% monocytes and T lymphocytes in the nonadherent low-density T lymphocyte–depleted population (Table 2). The titration curves of inhibition for rHF against colony formation by CFU-GM and BFU-E were similar as shown for one of two reproducible experiments using nonadherent low-density T lymphocyte–depleted human marrow as a target (Table 3). As shown in Table 3, the suppressive effects of rHF were similar whether the assays were performed in the presence of serum or in the presence of defined ingredients that were used to substitute for the serum.

DISCUSSION

The present studies demonstrating that rHF suppressed colony formation by human and mouse CFU-GM and human BFU-E substantiate our original observations that AIFs have a functional activity in vitro on myelopoiesis[5-9] and that homopolymers of liver H subunits are active and homopolymers of liver L subunits are inactive as suppressor molecules.[9] This information plus inactivation of the suppressive activity with monoclonal antibodies that recognize H chains and AIF demonstrates that it is the AIF itself, and not contaminating molecules, that is manifesting these effects.

Table 2. Comparative Influences of Purified rHF and rLF on Colony Formation by CFU-GM and BFU-E Progenitor Cells in Bone Marrow Containing or Depleted of Monocytes and T-Lymphocytes

	Colonies ($\bar{Y}$ ± 1 SEM)			
	CFU-GM		BFU-E	
Materials	LD BM	NALDT⁻ BM	LD BM	NALDT⁻ BM
Experiment 1				
Control medium	44 ± 3	30 ± 4	38 ± 4	44 ± 3
rHF (10^{-10} mol/L)	27 ± 2 (−38)¶	19 ± 1 (−38)**	27 ± 2 (−28)†	31 ± 3 (−30)§
Experiment 2				
Control medium	74 ± 3	31 ± 2	41 ± 3	63 ± 3
rHF (10^{-10} mol/L)	49 ± 3 (−33)#	23 ± 1 (−26)‡	31 ± 3 (−25)*	47 ± 2 (−26)*
Experiment 3				
Control medium	40 ± 3	32 ± 2	35 ± 5	32 ± 2
rHF (10^{-10} mol/L)	24 ± 2 (−40)‖	19 ± 2 (−41)‖	21 ± 2 (−40)‡	22 ± 2 (−38)§
rLF (10^{-9} mol/L)	39 ± 2 (−3)	33 ± 2 (+3)	32 ± 2 (−9)	32 ± 3 (0)

Low-density (LD) or nonadherent LD T lymphocyte–depleted (NALDT−) bone marrow (BM) cells were plated respectively at 10^5 or 5 × 10^4 cells/mL.

*Significant percent change from control, $P < .05$.

†$P < .04$.

‡$P < .03$.

§$P < .02$.

‖$P < .01$.

¶$P < .005$.

#$P < .002$.

**$P < .0001$.

The mechanism of action of AIF is not yet clear, but the action occurs in the absence of serum and after depletion of T lymphocytes and monocytes from the target marrow. Whether the rHF acts directly on the progenitor cells or indirectly via the few remaining T lymphocytes and monocytes or other accessory cells remains to be determined.

Our original report suggested that the inhibitory activity was associated with a glycosylated AIF molecule,[5] a result consistent with the recent report of Dezza et al.[15] However, since the rHF does not bind concanavalin A (Arosio, unpublished observations), it is clear that glycosylation of AIF is not an absolute prerequisite for functional activity. The rHF was active to concentrations as low as 10^{-10} to 10^{-11} mol/L. These are higher than the ones found active with our purified preparations of natural AIF.[5] It is possible that glycosylation may enhance the suppressive activity of AIF, and studies with rHF expressed in eukaryocytic cells may help to determine this. Alternatively, it is possible that greater activity may reside in a ferritin molecule containing mainly H subunits but with some L subunits, a fact we noted previously,[9] or a small contaminant in our purified preparations of natural AIF may have allowed for synergistic interactions, a phenomenon noted with other suppressor molecules.[34-37] This last alternative may not be true for all ferritin preparations since our original studies were done also with single bands of ferritin isolated from gels,[5] and we do not favor this last possibility.

Cukrova et al[17] have recently presented evidence that purified placental AIF has a suppressive activity in vitro. Placental AIF did not inhibit colony formation but did inhibit the entry of normal granulocyte-macrophage progenitor cells into S-phase. However, the placental CM used by Cukrova et al as a source of stimulating activity for their assay is not optimal for detecting suppression of colony

Table 3. Influence of Purified rHF and rLF on Colony Formation by CFU-GM and BFU-E Progenitor Cells in Nonadherent Low-Density T Lymphocyte–Depleted Bone Marrow Cultured in the Presence and Absence of Serum

	+ Serum		− Serum	
Materials	CFU-GM	BFU-E	CFU-GM	BFU-E
Control medium	249 ± 13	32 ± 2	108 ± 6	22 ± 2
10^{-10} mol/L rHF	183 ± 5 (−27)*	22 ± 2 (−38)†	71 ± 1 (−34)	15 ± 1 (−32)†
10^{-11} mol/L rHF	233 ± 8 (−6)	26 ± 2 (−19)‡	107 ± 7 (−1)	19 ± 2 (−14)
10^{-12} mol/L rHF	249 ± 11 (0)	30 ± 2 (−6)	110 ± 2 (+2)	22 ± 2 (0)
10^{-9} mol/L rLF	245 ± 13 (−2)	32 ± 3 (0)	109 ± 5 (+1)	21 ± 2 (−5)
10^{-10} mol/L rLF	245 ± 10 (−2)	31 ± 1 (−3)	111 ± 5 (+2)	20 ± 2 (−9)

Nonadherent low-density T lymphocyte–depleted bone marrow cells were plated at 5 × 10^4 cells/mL. CFU-GM numbers reflect colonies plus clusters.

*Significant percent change from control, $P < .01$.

†$P < .02$.

‡$P < .03$; other numbers are not statistically significant, $P > .05$.

formation by AIF since placental CM contains low but active levels of AIF that can partially or completely mask the colony-inhibiting effects of exogenously added AIF.[6]

The physiological and pathological relevance of AIF has been suggested,[5-9] but more information is needed to substantiate these beliefs. At present, the best evidence suggestive of a physiological role for AIF is that the administration of purified natural human AIF to mice mimics the effects of AIF in vitro.[8,9] Since human rHF can suppress colony formation by mouse marrow CFU-GM, it will be feasible to assess the effects of rHF in mice. The fact that human rHF was more active against human than mouse cells is consistent with our prior studies using purified preparations of natural human AIF[6] and suggests a degree of species specificity. Of relevance to a potential physiological role for AIF is that serum factors can bind AIF[38,39] as well as rHF (Arosio, unpublished observations) and that low or nondetectable levels of AIF in the serum have been reported and this may be due to the presence of serum-binding factors.

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REFERENCES

1. Harrison PM, White JL, Smith JMA, Farrants GW, Ford GC, Rice DW, Addison JM, Treffrey A: Comparative aspects of ferritin structure, metal-binding and immunochemistry, in Spik G, Montreuil J, Crichton RR, Mazurier J (eds): Proteins of Iron Storage and Transport. Amsterdam, Elsevier Biomedical, 1985, p 67

2. Aisen P, Listowsky I: Iron transport and storage proteins. Ann Rev Biochem 49:357, 1980

3. Arosio P, Adelman TG, Drysdale JW: On ferritin heterogeneity. Further evidence for heteropolymers. J Biol Chem 253:4451, 1978

4. Otsuka S, Maruyama H, Listowsky I: Structure, assembly, conformation and immunological properties of the two subunit classes of ferritin. Biochemistry 20:5226, 1981

5. Broxmeyer HE, Bognacki J, Dorner MH, deSousa M: The identification of leukemia-associated inhibitory activity (LIA) as acidic isoferritins: A regulatory role for acidic isoferritins in the production of granulocytes and macrophages. J Exp Med 153:1426, 1981

6. Broxmeyer HE, Bognacki J, Ralph P, Dorner MH, Lu L, Castro-Malaspina H: Monocyte-macrophage derived acidic isoferritins: Normal feedback regulators of granulocyte-macrophage progenitor cells. Blood 60:595, 1982

7. Lu L, Broxmeyer HE, Meyers PA, Moore MAS, Thaler HT: Association of cell cycle expression of Ia-like antigenic determinants on normal human multipotential (CFU-GEMM) and erythroid (BFU-E) progenitor cells with regulation in vitro by acidic isoferritins. Blood 61:250, 1983

8. Broxmeyer HE, Gentile P, Cooper S, Lu L, Juliano L, Piacibello W, Meyers PA, Cavanna F: Functional activities of acidic isoferritins and lactoferrin in vitro and in vivo. Blood Cells 10:397, 1984

9. Broxmeyer HE, Gentile P, Listowsky I, Cavanna F, Feickert HJ, Dorner MH, Ruggeri G, Cazzola M, Cooper S: Acidic isoferritins in the regulation of hematopoiesis in vitro and in vivo, in Albertini A, Arosio P, Chiancone E, Drysdale J (eds): Ferritins and Isoferritins as Biochemical Markers. Amsterdam, Elsevier, 1984, p 97

10. Taetle R: Acidic isoferritins (leukemia-associated inhibitory activity) fail to inhibit blast proliferation in acute myelogenous leukemia. Blood 58:653, 1981

11. Pelus LM: Association between colony forming units' granulocyte macrophage expression of Ia-like (HLA-DR) antigen and control of granulocyte and macrophage production. A new role for prostaglandin E. J Clin Invest 70:568, 1982

12. Sala G, Worwood M, Jacobs A: The effect of isoferritins on granulopoiesis. Blood 67:436, 1986

13. Broxmeyer HE: Letter to the editor. Reply to Jacobs: Blood 62:702, 1983

14. Broxmeyer HE: Comments on article of Sala et al. Letter to the editor. Blood 68:794, 1986

15. Dezza L, Cazzola M, Piacibello W, Arosio P, Aglietta M: Effect of acidic and basic isoferritins on the in vitro growth of human granulocyte-monocyte progenitors. Blood 67:789, 1986

16. Moore RN, Joshi JG, Deanna DG, Pitruzzello FJ, Horohov DW, Rouse BT: Characterization of a two-signal–dependent, Ia^+ mononuclear phagocyte progenitor subpopulation that is sensitive to inhibition by ferritin. J Immunol 136:1605, 1986

17. Cukrova V, Hrkal Z, Koprivova H, Neuwirt J: Identification of leukemia cell–derived inhibitory activity (LIA) in conditioned media from human myeloid leukemia cell line ML-2. Blut 52:51, 1986

18. Boyd D, Jain SK, Crampton J, Barrett KJ, Drysdale J: Isolation and characterization of a cDNA clone for human ferritin heavy chain. Proc Natl Acad Sci USA 81:4751, 1984

19. Costanzo F, Santoro C, Colantuoni V, Bensi G, Raugei G, Romano V, Cortese R: Cloning and sequencing of a full length cDNA coding for a human apoferritin H chain: Evidence for a multigene family. EMBO J 3:23, 1984

20. Boyd D, Vecoli C, Belcher DM, Jain SK, Drysdale JW: Structural and functional relationships of human ferritin H and L chains deduced from cDNA clones. J Biol Chem 260:11755, 1985

21. Costanzo F, Colombo M, Staempflis S, Santoro C, Marone M, Frank R, Delius H, Cortese R: Structure of gene and pseudogenes of human apoferritin H. Nucleic Acids Res 14:721, 1986

22. Arosio P, Levi S, Albertini A, Ruggeri G, Iacobello C, Luzzago A, DeSimone F, Cesareni C. Cortese R: Development of monoclonal antibodies and recombinant human ferritins for structural analysis of isoferritins, in Koprowski H, Ferrone S, Albertini A (eds): Biotechnology in Diagnosis. New York, Elsevier, 1985, p 237

23. Levi S, Cesareni G, Arosio P, Lorenzetti R, Sollazzo M, Albertini A, Cortese R: Characterization of human ferritin H chain synthesized in *E coli*. Gene (in press)

24. Dorner MH, Salfeld J, Will H, Leibold EA, Vass JK, Munro HN: Structure of human ferritin light subunit messenger RNA: Comparison with heavy subunit message and functional implications. Proc Natl Acad Sci USA 82:3139, 1985

25. Salfeld J: Doctoral Thesis, University of Heidelberg, Germany, 1985

26. Broxmeyer HE, Juliano L, Lu L, Platzer E, Dupont B: HLA-DR human histocompatability leukocyte antigen–restricted lymphocyte-monocyte interactions in the release from monocytes of acidic isoferritins that suppress hematopoietic progenitor cells. J Clin Invest 73:939, 1984

27. Remaut E, Stannssens P, Fiers W: Plasmid vectors for high efficiency expression controlled by the P_L promoter of coliphage lambda. Gene 15:81, 1981

28. Luzzago A, Arosio P, Iacobello C, Ruggeri G, Copucci D, Brochi E, DeSimone F, Gamba D, Gabri E, Albertini A: Immunochemical characterization of human liver and heart ferritins with monoclonal antibodies. Biochim Biophys Acta 872:61, 1986

29. Cavanna F, Ruggeri G, Iacobello C, Chieregatti G, Murador E, Albertini A, Arosio P: Development of a monoclonal antibody against human ferritin and its application in an immunoradiometric assay. Clin Chim Acta 134:347, 1983

30. Arosio P, Levi S, Gabri E, Ruggeri G, Iacobello C, Albertini A, Cavanna F: Heterogenity of ferritin, II: Immunological aspects, in Albertini A, Arosio P, Chiancone E, Drysdale JW (eds): Ferritins and Isoferritins as Biochemical Markers. Amsterdam, Elsevier, 1984, p 33

31. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680, 1970

32. Hames BD: Introduction to PAGE, in Hames BD, Rickwood D (eds): Gel electrophoresis of proteins, a practical approach. Washington, DC, IRL, p 74

33. Lu L, Broxmeyer HE: Comparative influences of phytohemagglutinin stimulated leukocyte conditioned medium, hemin, prostaglandin E and low oxygen tension on colony formation by erythroid progenitor cells in normal bone marrow. Exp Hematol 13:989, 1985

34. Broxmeyer HE, Cooper S, Rubin BY, Taylor MW: The synergistic influence of human interferon-γ and interferon-α on suppression of hematopoietic progenitor cells is additive with the enhanced sensitivity of these cells to inhibition by interferons at low oxygen tension in vitro. J Immunol 135:2502, 1985

35. Raefsky EL, Platanias LC, Zoumbos NC, Young NS: Studies of interferon as a regulator of hematopoietic cell proliferation. J Immunol 135:2507, 1985

36. Broxmeyer HE, Williams DE, Lu L, Cooper S, Anderson SL, Beyer GS, Hoffman R, Rubin BY: The suppressive influences of human tumor necrosis factor on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: Synergism of tumor necrosis factor and gamma interferon. J Immunol 136:4487, 1986

37. Degliantoni G, Murphy M, Kobayashi M, Francis MK, Perussia B, Trinchieri G: Natural killer (NK) cell–derived hematopoietic colony inhibiting activity and NK cytotoxic factor. Relationship with tumor necrosis factor and synergism with immune interferon. J Exp Med 162:1512, 1985

38. Covell AM, Worwood M: Isoferritins in plasma, in Albertini A, Arosio P, Chiancone E, Drysdale J (eds): Ferritins and Isoferritins as Biochemical Markers. Amsterdam, Elsevier, 1984, p 49

39. Cazzola M, Arosio P, Bellotti V, Gaetano B, Dezza L, Iacobello C, Ruggeri G: Use of a monoclonal antibody against heart ferritin for evaluating acidic ferritin concentration in human serum. Br J Haematol 61:445, 1985

Leukemia Diagnosis and Testing of Complement-Fixing Antibodies for Bone Marrow Purging in Acute Lymphoid Leukemia

Dario Campana and George Janossy

In this paper a microplate method is described for diagnosing acute leukemia and for investigating the reactivity of monoclonal antibodies (MoAbs) against membrane antigens in combination with rabbit or murine antibodies to nuclear terminal transferase (TdT). The speed of this method facilitates the investigation of fresh leukemic cells from individual patients and assesses the cytolytic efficacy of the relevant MoAbs in the presence of complement (C′). Lymphoblasts (TdT+) are mixed in equal proportions with known numbers of "inert" cells, eg, RBC or nonleukemic bone marrow (BM). Following incubation with MoAbs and C′ the ratio of residual TdT+ cells and inert cells is determined on cytospin preparations. Initially, percentages of TdT+ cells are counted in a unit volume of 5,000 inert cells, followed by the scanning of $>2 \times 10^4$ inert cells on entire slides. With this method more than 4 log cytoreduction of TdT+ cells is detected. The method is also applicable for studying the cytolysis of malignant B cells by using mostly monoclonal Ig expression rather than TdT for the identification of residual B cells. Ten representative patients selected from a group of >100 are reported. In some cases cytoreduction of >4 log with no identifiable residual TdT+ cells is achieved by a single C′-fixing MoAb: anti-CD10 (RFAL3) in common acute lymphoid leukemia (ALL) and anti-CD7 (RFT2) in T cell ALL (T-ALL). Other cases require cocktails of anti-CD10, anti-CD19, and anti-CD24 in common ALL or anti-CD7 and anti-CD8 in T-ALL. In T-ALL a few TdT+ cells remain that exhibit the features of normal TdT+ BM cells (CD7$^-$, HLA-DR$^+$). This is particularly noticeable when patients are studied in partial remission or if nonleukemic BM is used as a source of inert cells. The methods described here contribute to establishing a range of MoAbs (ie, of IgM class) and techniques for efficient purging and to comparing the efficacy of "clean-up," in remission, of common ALL, T-ALL, and B cell malignancies.

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THE ELIMINATION of unwanted cells from the bone marrow (BM) during bone marrow transplantation (BMT) is referred to as "purging" or "cleansing." Monoclonal antibodies (MoAbs) able to fix complement (C′)[1,2] or toxin conjugates[3] have been used successfully to remove 95% to 99.5% of T lymphocytes from allogeneic BM in order to prevent graft-*v*-host disease.[4] Similar principles can also be applied for eliminating residual leukemia/lymphoma from the BM prior to autologous BMT.[5-8] During this procedure proof is required to show the efficient removal of the malignant clone.

Conventional methods for investigating the efficacy of C′ lysis such as dye exclusion and ^{51}Cr release are unsuitable for detecting small numbers of residual viable malignant cells. Flow cytometry, when performed with only one single antibody, also lacks the necessary sensitivity as its lower limit of detection is 0.5% to 1%.[9] During the last 3 years ingenious clonogenic assays have also been developed showing that MoAbs with C′ or immunotoxins are capable of destroying 3 to 6 logs of cells taken from permanent cell lines of Burkitt[10,11] or acute lymphoid leukemia (ALL) origin[12-14] and added as contaminants to suspensions of normal BM.[12-14] Nevertheless, these model studies do not yield information about the heterogeneity of fresh leukemic cells in terms of their suspectibility to Ab-mediated cytopathic effects.

Two aspects of purging need to be assessed when leukemic cells from patients are studied with a clinical program in mind. One is the exact proportion of leukemic cells that react with an antibody (or cocktail). The other is the efficacy of a particular cytolytic, toxic, or separating method in eliminating the population of positively labeled leukemic cells. In order to answer both of these questions, additional markers independent of the MoAb(s) used for purging have to be applied to detect leukemia. These may include (1) cytology, for the morphology of leukemic blasts such as B-ALL cells of the L3 category can be very characteristic even when seen in minute proportions; (2) detection of malignancy by chromosomal or DNA abnormality[15]; (3) expression of membrane markers studied by additional MoAbs or by antiimmunoglobulin (anti-κ, anti-λ, or antiidiotype) Abs in B cell disorders; and finally, (4) a nuclear enzyme, terminal deoxynucleotidyl transferase (TdT) in ALL and related disorders.[16]

In this paper we standardize methods suitable for quantitating residual ALL blast cells. While approaching this aim, three steps have been introduced: (1) A microplate method[17] with multiple-sample handling has been adopted for rapid immunodiagnosis. (2) Murine anti-TdT MoAbs have been employed[18] and used in double immunofluorescence (IF) combinations with MoAbs directed against membrane antigens. (3) A quantitative light microscopic test has been developed for assessing the efficacy of C′ lysis in leukemia by both immunologic (TdT+) and morphological criteria. In this test leukemic cells are mixed with inert cells (eg, red cells) in known proportions. C′ lysis is performed, and the loss of leukemic cells is determined following TdT staining in cytospins. When the proportions of TdT+ (leukemic) *v* inert cells are counted in the control (untreated) and in post-C′ samples, the depletion of leukemic (TdT+) blasts is precisely evaluated.

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MATERIALS AND METHODS

Handling of cells. Sterile samples from peripheral blood (PB) and BM were separated on a Ficoll-Hypaque gradient. Mononuclear cells were washed twice in phosphate-buffered saline (PBS) and reconstituted at 2 to 4 × 10^6/mL in PBS containing 0.2% human serum albumin and 0.2% azide (PBSA). The cells prepared for C′ lysis were reconstituted at 2 × 10^7/mL in Hanks' balanced salt solution (HBSS with Ca^{++} and Mg^{++}, GIBCO, Paisley, UK). Cytospins were made in a Shandon cytocentrifuge (Southern Products, Astmoor, UK), fixed in cold methanol for 30 minutes, and washed in PBS. After incubation with Abs, cytospins were covered with a 1:1 mixture of glycerol and PBS or with a medium of polyvinyl alcohol and diazobicyclooctane, which sets and retards fading.[19] Other slides were stained with May-Grünwald-Giemsa.

The patients' RBC were washed four times in PBS and resuspended in HBSS. Chicken RBCs (Tissue Culture Services, Slough, UK; catalog no. 101010) were used fresh or fixed in 4% formalin. The latter were washed in PBS with 5% bovine serum albumin (BSA) and stored sterile at 4 °C until use. The HL-60 cell line was grown in RPMI 1640 medium containing 10% fetal calf serum. The cells were washed three times in PBS and resuspended at a 2 × 10^7 concentration in HBSS.

MoAbs. A standard panel of 12 MoAbs was used to detect membrane antigens[17] (Table 1). Stem cell–associated MoAbs were BI-3C5[20] and anti–HLA-DR. The myeloid MoAbs were My-9[28] or MCS-2[29] and UCHM1—an Ab reactive with monocytes and (myelo)monocytic leukemia.[21] The common ALL (cALL) and B lymphocyte reagents were Abs to CD19, CD10 (cALL), CD20-like antigen, and IgM as defined at the 2nd Workshop on Leukocyte Differentiation Antigens (LDA), Boston.[22] The anti-T reagents were Abs to CD7, CD2, CD3, and CD5 as defined in the 1st Workshop on LDA, Paris.[23,24]

The four MoAbs made against purified human TdT (HTdT-1, HTdT-2, HTdT-3, and HTdT-4, all of IgG1 subclass) were provided by Professor F.J. Bollum.[18] The mixed cocktail of these four Abs is referred to as HTdT (total Ab content, 1 mg/mL containing approximately 0.25 mg of each Ab per milliliter). HTdT was also conjugated with biotin.[25]

Two MoAbs used for purging were of IgM class and fixed both rabbit and human C′. These were RFAL-3 reacting with CD10, cALL antigen, and RFB7 reacting with a CD20-like B cell–specific antigen.[26] RFT2 (anti-CD7) was of the IgG2 class[24] and fixed only rabbit C′. Additional reagents such as Abs to CD19, CD24, and CD8 were also used in the various cocktails; these were SB4,[27] BA1[30] and RFT8,[24] respectively, all of which were of the IgM class.

Heterologous antisera. Fluorochrome-labeled goat antisera to human IgM (G-anti-H-IgM–tetramethyl rhodamine isothiocyanate [TRITC], catalog no. 2020-03), κ and λ light chains (G-anti-H-κ-TRITC, catalog no. 2060-03; and G-anti-H-λ–fluorescein isothiocyanate (FITC), catalog no. 2070-02), as well as to mouse IgG and IgM (G-anti-M-IgG-FITC, catalog no. 1030-02; and G-anti-M-IgM-TRITC, catalog no. 1020-03) were purchased from Southern Biotechnology Associates, Birmingham, AL. These reagents were heavy or light chain specific, respectively. Goat antimouse Ig was eluted from an M-Ig column and conjugated with FITC or TRITC in our laboratory (G-anti-M-Ig-FITC or -TRITC) and had no activity against goat, human, rabbit, or swine Ig.

Rabbit antisera to calf TdT (R-anti-TdT; Supertech Inc, Bethesda, MD, catalog no. 004) reacted with HTdT in the nucleus of thymocytes and rare non-T, non-B cells of the normal human BM, but were negative (<0.1%) on circulating blood lymphocytes as well as on lymphoid cells of the human palatal tonsil. Swine antirabbit Ig (Sw-anti-R-Ig-TRITC) was from Dakopatt, Copenhagen (catalog no. R156) and had no activity against goat, human or mouse Ig.

Table 1. Diagnostic Reagents in Leukemia

			Reactivity Pattern						
Ab (Feature)	Equivalent Local Ab	mol wt (Thousands)	cALL	AML	T-ALL	Peripheral T-Leukemia	B-NHL	B-CLL	Reference
Stem cell associated									
1. BI-3C5		120	+	+	–	–	–	–	20
2. Class II	RFDR1	28, 33	+	+ (–)*	–	–	+	+	
Myeloid									
3. My9, MCS2			–	+	–	–	–	–	28, 29
4. UCHM1		52	–	some +	–	–	–	–	21
Common ALL and B									
5. anti-CD19		94	+	–	–	–	+	+	22, 27
6. anti-CD10	RFAL3	100	+	–	– (±)†	–	–/+	–	5, 22
7. anti-CD20	RFB7	35	–	–	–	–	+	+	7, 22
8. anti-IgM			–	–	–	–	+	+	
T cell associated									
9. anti-CD7	RFT2	40	–	– (±)‡	+	+ or –§	–	–	23
10. anti-CD2 (T11)	RFT11	50	–	–	+	+	–	–	23
11. anti-CD3 (T3)		19–29	–	–	–‖	+	–	–	23
12. anti-CD5 (T1)	RFT1	65	–	–	+	+¶	–	+#	23
TdT			+	–	+	–	–	–	16, 18

The reagents were used in a microplate assay system. On the basis of the results additional Abs are used for further analysis.

Abbreviations: AML, acute myelogenous leukemia; B-NHL, B cell non-Hodgkin's leukemia; T-CLL, T cell chronic lymphocytic leukemia.

*AMLs are heterogeneous in terms of HLA-DR expression.

†A few T-ALL cases weakly express cALL antigen.

‡Some AMLs carry p40 (CD7) antigen.

§Sézary cells are p40 (CD7)-negative.

‖A few T-ALL cases are weakly membrane CD3 (T3)-positive (but many show cytoplasmic CD3).

¶T-CLL is frequently CD5 (T1)-negative.

#B-CLL carries CD5 (T1) antigen.

Avidin was bought from Sigma Chemical Co, St Louis (catalog no. A-3275) and conjugated to TRITC.

Double IF assays. Leukemic blasts were labeled in suspension with MoAbs using FITC-conjugated second layers. Then cytospins were made and fixed in cold methanol. The cells were restained with anti-TdT and TRITC-conjugated second layer. Four assays were studied: (1) MoAb + G-anti-M-Ig-FITC in combination with R-anti-TdT + Sw-anti-R-Ig-TRITC[31]; (2) MoAb of the IgM class + G-anti-M-IgM-TRITC in combination with HTdT (IgG) + G-anti-M-IgG-FITC; (3) MoAb + G-anti-M-Ig-FITC–labeled cells cytocentrifuged and then incubated with normal mouse serum (in order to block binding to G-anti-M-Ig) followed by biotinated HTdT Ab + avidin-TRITC; and (4) the same as 3 except that HTdT was followed by G-anti-M-Ig-TRITC (Fig 1).

Microplate method. Fifty-microliter aliquots of a 2 to 4 × 10^6/mL suspension (1 to 2 × 10^5/well) were placed into 12 microwells in a U-bottomed microplate (Sterilin, UK; catalog no. M24A) with a repeating dispenser (2 mL total volume; Jencons, UK; catalog no. H9/255-22). Twelve (2 × 6) MoAbs (Table 1) kept in an adjacent plate as a row of concentrated stock solution and diluted with PBSA prior to use were transferred in 50-μL volumes onto the cells with a Titertek multipipette (using six of the eight available channels; Flow Labs, UK; catalog no. 77-869-00). The individual wells on the whole plate were covered with an adhesive sheet and gently shaken while incubating for ten minutes at 20 °C. The wells were topped up with 100 μL PBSA, spun in a centrifuge (Beckman TJ-6 with plate carriers) for 30 seconds at 1,500 rpm, and the supernatant was removed by inverting the plate. The cells were resuspended on a plate shaker and washed four times with cold PBSA. Cells were then incubated again for ten minutes with diluted G-anti-M-Ig-FITC (50 μL) and washed four times. After the last wash, 5 to 6 μL PBSA (with azide) was added to each of the small cell pellets using a smaller Titertek multipipette (catalog no. 77-858-00), and 2 μL of resuspended cells were transferred, as two rows of six samples, onto a polytetrafluoroethylene (PTFE)-coated multispot slide (catalog no. PH001; Hendley, Essex, UK). One slide (12 samples) for each patient was placed into formalin vapor for ten minutes, dried, and covered.[17]

These first results identified which of the 12 MoAbs had reacted with the largest proportions of blast cells. Cells already membrane labeled were then lifted from some selected relevant wells in order to restain blasts with TdT- and TRITC-conjugated second layers.

Standardization of C′. Thirty-day rabbit-C′ (TDRC) was obtained from 1-month-old rabbits (Research Sera Ltd, UK). In the presence of anti-T cell MoAbs of the IgM class, a cocktail of CD6 (RFT12) and CD8 (RFT8) TDRC used at 33% final dilution destroyed >98% of the T lymphocytes. This demonstrated the lytic power of the C′ batch. At the same time B lymphocytes or myeloid colony-forming cells were not affected, indicating the lack of nonspecific toxicity.[4] Batches of TDRC were kept at −70 °C.

RESULTS

Performance of anti-TdT MoAbs. MoAbs to TdT (HTdT-1, -2, -3, and -4) and R-anti-TdT were studied on suspensions of normal BM (three samples), tonsil (five samples), thymus (three samples) and PB (five samples). All four MoAbs and R-anti-TdT showed identical reactivity: 2% to 3% positivity in BM, 59% to 77% in thymus, and no positive cells in tonsil and PB. When tested on ALL (14 cases), all four MoAbs gave positive staining: HTdT-1 showed strong, HTdT-3 and -4 moderate, and HTdT-2 weak nuclear labeling. The combination of all four (HTdT) reagents gave the strongest staining, comparable to R-anti-TdT. HTdT did not show positivity on seven cases of AML (My-9+ and TdT− when tested with R-anti-TdT).

In the next experiments R-anti-TdT was used in double combination with HTdT. In suspensions of thymocytes (two samples), normal BM (two samples), and ALL (five cases), widely different numbers of positive cells (2% to 3% in normal BM and >60% reactivity in thymus and ALL) were seen, and still the two reagents showed concordant staining on all nuclei (>1,000 cells scanned in each sample). The R-anti-TdT showed stronger cytoplasmic staining on some

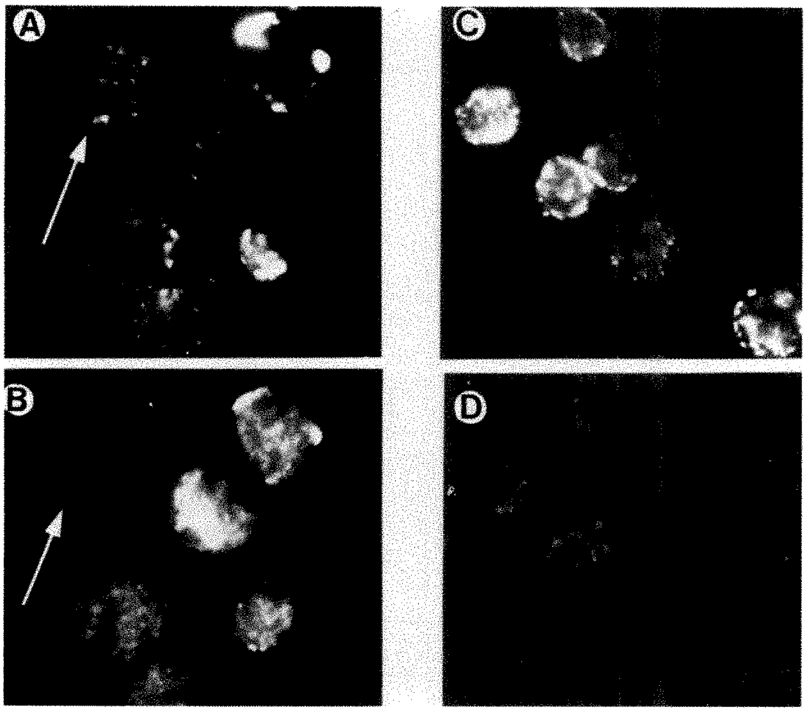

Fig 1. Double labeling of membrane antigens and TdT using combination as follows: membrane staining in suspension with MoAb and G-anti-M-Ig-FITC and restaining the cytospins with HTdT and G-anti-M-Ig-TRITC (see the text). Common ALL blast cells (A, B) are labeled for CD10 antigen (RFAL-2 in A) and TdT (B). Blood lymphocytes are labeled for T3 antigen (CD3, in C), but TdT− (D). On the FITC channel the membrane labeling is specific (A, C). On the TRITC channel nuclear TdT staining is seen on ALL blasts (B), and there is weak clear membrane cross-reaction (B, D). The arrow points to a CD10+, TdT− blast.

myelocytic-granulocytic cells than HTdT, but this weak extra staining did not interfere with the evaluation of results. Previous studies have already revealed the correlation between nuclear TdT staining and TdT enzyme activity measured by biochemical assay in ALL and AML[32] and also showed the irrelevance of weak cytoplasmic TdT staining.

In the next experiment three combinations for membrane and nuclear labeling were tested (see Methods). In combination 2, the strong labeling with RFAL-3 (anti-CD10; IgM class + G-anti-M-IgM-TRITC) did not interfere with HTdT visualized by G-anti-M-IgG-FITC (heavy chain specific). In combination 3, RFAL-2 (anti-CD10, IgG2 class) and HTdT-biotin showed no membrane-nuclear cross-reactivity, and the nuclear HTdT-biotin plus avidin-FITC staining was again strong. The individual anti-TdT Abs did not perform as well as the HTdT cocktail.

Combination 4 was also satisfactory. A clear membrane positivity was seen on ALL blasts using RFAL-2 (anti-CD10) and G-anti-M-Ig-FITC (Fig 1A). HTdT + G-anti-M-Ig-TRITC revealed a strong nuclear TdT staining in ALL blasts (Fig 1B) and only a weaker additional membrane labeling on the TRITC channel (Fig 1B and D). These findings demonstrate the feasibility of double membrane/TdT staining using MoAbs to TdT.

Standardization of artificial mixtures of cells. After having demonstrated the suitability of MoAbs to TdT, tests were set up to investigate the efficacy of C′ lysis in leukemic samples taken at presentation. Cells (10^6 cells in 50 μL HBSS) were placed into U-bottomed microwells, and MoAbs as culture supernatants were added in 50-μL volumes at saturating conditions and incubated for 15 minutes at 20 °C. The supernatant was discarded, and rabbit serum diluted in HBSS as source of C′ was added for 45 minutes at 37 °C followed by a second round of C′. Cells were then washed twice in PBSA, incubated with G-anti-M-Ig-FITC for ten minutes, and washed, and multiple cytospins were made for R-anti-TdT staining. More recently, MoAb combination 2 with class-specific second layers was used. Cytospins for May-Grünwald-Giemsa staining were also prepared.

When the C′ lysis was efficient and high proportions of ALL blast cells were destroyed, virtually no cells could be recovered for analysis. Thus two modifications were introduced: (1) During the last ten minutes of incubation with both rounds of C′, DNAse (10 U/well, Sigma, UK; catalog no. D0751) was added. This solubilized DNA in the debris and prevented clumping. (2) Known numbers of inert cells were mixed into the sample. These remained viable during C′ lysis, and carried over residual cells onto the cytospin slides even when these were present only in small numbers. Second, inert cells rendered the test quantitative (see the following material).

We have investigated possible errors such as selective loss or accumulation of certain cell types known to occur during cytocentrifugation. Suspensions of BM, HL-60, human RBC, and chicken RBC were mixed with ALL blasts in near equal numbers and cytocentrifuged (Table 2). ALL blasts appeared to be slightly diluted out (19.9% and 17.5% ALL loss) by the accumulating larger myeloid cells in the BM and by the HL-60 cells. As a contrast, ALL blasts were apparently enriched (22% enrichment) due to the relative loss of chicken RBC. This phenomenon was seen with both the fresh and formalin-fixed chicken RBC and could not be attributed to RBC lysis by heterophil antiavian antibodies present in the rabbit serum. Only minor changes (7% ALL enrichment) were, however, seen in ALL/human RBC mixtures, and for this reason we adopted human RBC inert cell populations with additional controls when normal BM was used (Table 3, patients 3b and 6b).

The results of the C′ lysis assay were expressed as follows. First, the numbers of TdT+ cells were counted in an area of slides containing 5,000 inert cells (human RBC) after treatment with relevant cytolytic MoAb + C′. This value is referred to as N1. Next, the TdT+ cells were counted per 5,000 inert cells after treating an aliquot of the same suspension with an irrelevant, ineffective MoAb + C′. This value is referred to as N2. The N1/N2 ratio gives the percentage of surviving TdT+ cells. After this counting, in samples with <0.02% residual leukemia the whole slide and an additional duplicate cytospin preparation of the same C′-treated sample were scanned and residual TdT+ cells, if any, recorded among $>2 \times 10^4$ RBC. Thus this method is well suited for studying >4 log cytoreduction.

Additional controls were also performed. These were proportions of TdT+ cells per 5,000 inert cells incubated in the absence of MoAb or in the absence of C′. Finally, the study concluded by recording the following parameters: (1) proportions of TdT+ cells with membrane labeling (inefficiency of C′, Fig 2C), (2) proportions of residual TdT+ blasts without membrane labeling (lack of Ab cover, Fig 2D), and (3) proportion of residual TdT− blasts (in cases of mixed lymphoid/myeloid leukemia, Fig 2E).

Purging with MoAbs and rabbit C′. In Table 3 typical observations are shown from ten patients with ALL selected from >100 cases. Patients 1 to 4 had common ALL based on the results of microplate assay and the TdT test: BI-3C5$^+$, HLA-DR$^+$, CD19$^+$, CD10$^+$, and TdT$^+$, but essentially

Table 2. Relative Changes in the Proportion of ALL Blast Cells Due to Cytocentrifugation *

No. of ALL/Inert Cells	Experiment 1	Experiment 2	Experiment 3	Mean	Percentage
ALL/normal BM	76.9 ± 2.5	83.2 ± 8.8	—	80.1	−19.9
ALL/HL60	81.1 ± 6.7	91.5 ± 9.3	75.0 ± 9.1	82.5	−17.5
ALL/human RBC	111.1 ± 4.1	104.9 ± 5.1	105.8 ± 6.1	107.2	+7.2
ALL/chicken RBC	119.3 ± 6.4	124.4 ± 9.6	122.7 ± 10.0	122.1	+22.1

*Figures are the percentages of ALL blasts related to a standard number of inert cells (hemopoietic, HL60, human or chicken RBC) following cytocentrifugation. The ALL/inert cell value in suspension prior to spinning was 100%. Usually 1,600 inert cells were counted in each of the eight samples (±SEM).

Table 3. Elimination of Leukemic Cells with Ab + C′

	Patient											
	1	2	3		4	5	6		7	8	9	10
Diagnosis	cALL	cALL	cALL		cALL	null ALL	T-ALL		T-ALL	T-ALL	mixed ALL	B-ALL
Blasts in sample (%)	97	95	97		48	95	93		98	80	95	88
Ab reactivity												
HLA-DR (RFDR1)	95	96	94		59	97	5		2	11	96	91
CD10 (RFAL3)	82	94	83		17	<1	70±		<1	<1	34	<1
CD7 (RFT2)	3	2	5		34	2	94		97	83	1	2
CD20 (RFB7)	1	1	1		5	<1	<1		<1	1	<1	90
TdT (HTdT)	87	83	91		42	95	84		77	78	31	<1
Combined complement lysis and TdT test			3a	3b			6a	6b				
N1 (relevant Ab)	<1 (RFAL3)	189 (RFAL3)†	72 (RFAL3)‡	36 (RFAL3)‡	3,200 (RFAL3)	4,850§ (RFAL3)	<1 (RFT2)	90‖ (RFT2)	66¶ (RFT2)	5‖ (RFT2)	66 (RFAL3)	<1 (RFB7)
N2 (irrelevant Ab)	4,200 (RFT2)	5,100 (RFT2)	5,000 (RFT2)	3,600 (RFT2)	4,500 (RFT2)	3,000 (RFT2)	5,100 (RFB7)	3,500 (RFB7)	4,700 (RFB7)	5,000 (RFB7)	2,000 (RFB7)	4,900 (RFT2)
N1/N2 (%)	<0.01*	3.7†	1.4‡	1.0‡	71	87§	<0.01*	<0.02‖	1.4¶	<0.02‖	3.3#	<0.01**

*When the whole slide was scanned (>10,000 RBCs) no residual TdT+ cells were seen (>4 log kill).

†The residual blasts were RFAL3⁺ (Fig 2C) and were fully destroyed by a fresh batch of rabbit C′ (see text; <0.01% TdT⁺ cells).

‡These blasts were RFAL3− (Fig 2D), and additional CD19 (SB4) was required to obtain a >4 log kill.

§CD24 (BA1) Ab was efficient in removing these blast cells.

‖The few residual TdT+ cells were RFT2−, HLA-DR+ normal BM precursor cells. No TdT+, HLA-DR−, T-ALL blasts were seen in >10,000 RBC (>4 log specific kill).

¶Removed by RFT2 + RFT8 (CD7 + CD8). N1/N2 < 0.03. No residual TdT+ in >10,000 RBC (>4 log kill).

#Residual additional TdT− myeloblasts: 71% (Fig 2E).

**When the whole slide was scanned (>10,000 RBCs) no residual IgM+, λ+ cells were seen (>4 log kill).

negative with other MoAbs listed in Table 1. Patient 1 had no remaining identifiable TdT+ cells per 5,000 admixed RBC following incubation with RFAL-3 and C′ (N1:<1), but had 4,200 TdT+ cells per 5,000 RBC (N2) following incubation with an irrelevant, unreactive MoAb (anti-CD7) and C′. The scanning of slides revealed no TdT+ cells among >2 × 10⁴ RBC (>4 log kill). The morphological analysis of purged cells showed myeloid cells, plasma cells, and small- and intermediate-sized lymphocytes of the T3⁺, TdT− type (normal T cells). In Patient 2 the first analysis revealed residual TdT+ cells following incubation with RFAL-3 and C′ (N1: 189 per 5,000 RBC in the treated aliquot) and 5,100 TdT+ cells per 5,000 RBC in the control (N2). This corresponds to 3.7% residual disease (N1/N2). This latter population expressed CD10 antigen strongly (Fig 2C), pointing to suboptimal C′ activity. The experiment was repeated with a fresh C′ sample, and the blast cell lysis was now efficient (>4 log kill).

Patient 3 had a small population of residual TdT+ cells (N1, 72; N1/N2, 1.4%) but these were CD10⁻. The TdT+

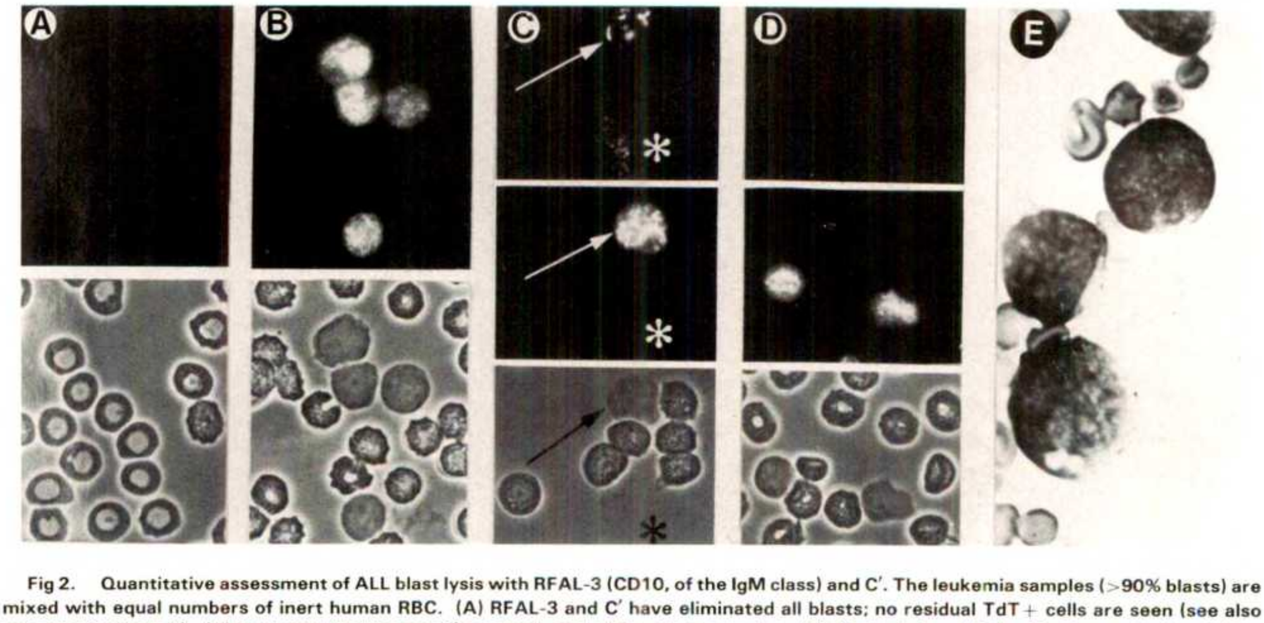

Fig 2. Quantitative assessment of ALL blast lysis with RFAL-3 (CD10, of the IgM class) and C′. The leukemia samples (>90% blasts) are mixed with equal numbers of inert human RBC. (A) RFAL-3 and C′ have eliminated all blasts; no residual TdT+ cells are seen (see also patient 1 in Table 3). (B) Irrelevant MoAbs and C′ are used, and TdT+ blasts survive. (C) The lysis is not fully effective, and a few TdT+ cells remain that carry CD10 (cALL antigen, see arrow; from patient 2). The asterisk shows a dead lysed blast. (D) The residual TdT+ cells lack RFAL-3 (CD10) reactivity (patient 3). (E) A mixed leukemia is shown. The blast cells are not lysed by RFAL-3: TdT−, myeloid blasts (patient 9 in Table 3). Photographs are double (A, B) or triple (C, D) exposures of the same field with IF and phase contrast.

blasts were not clearly identifiable by morphology and masqueraded as lymphocytes. The purging was repeated with a mixture of MoAb RFAL-3 (anti-CD10) and SB4 (anti-CD19), and no identifiable TdT+ cells were left (>4 log kill). Identical results were seen when instead of human RBC normal bone marrow was the source of added inert cells (patient 3b in Table 3). These results emphasize the need for using cocktails.

A sample of PB taken from patient 4 in early relapse had a mixture of normal T lymphocytes (T3+, HLA-DR−, TdT−) and leukemia (TdT+, HLA-DR+, T3−), identified in T3/TdT and HLA-DR/TdT double-marker assays. The TdT+ blast cells were heterogeneous: only 40% of these were CD10$^+$. Only this subset of blast cells was lysed by CD10 (N1, 3,200; N1/N2, 71%). By adding further Abs to CD19, CD9, and CD24, larger percentages of TdT+ blasts were eliminated, but a few always remained (N1/N2, >10%). We could not find a fully efficient Ab cocktail for purging the leukemia of patient 4.

Patient 5 had a cALL antigen–negative null ALL (CD10, totally negative; HLA-DR+, TdT+). Consequently, the incubation with RFAL-3 was ineffective (N1/N2, 97%). On the other hand, the blast cells were CD19+, CD24+. Good lysis (>4 log cytoreduction) was achieved in this patient with anti-CD24 (BA1 MoAb).[30]

Patients 6 to 8 had T-ALL: CD7$^+$, mostly CD2$^+$, and essentially negative with MoAbs detecting other non-T antigens (except a variable CD10 expression, see patient 6). Following the incubation with RFT2 (anti-CD7) and C′, in all three cases a drastic reduction of TdT+ cells was observed. In patient 6 the lysis of TdT+ cells was complete (>4 log). When the test was repeated (in 6b) using admixed normal BM, 2.6% residual TdT+ cells were seen, but these were all HLA-DR+, CD7− ($>10^2$ TdT+, HLA-DR+ cells counted). It is therefore concluded that the specific lysis of T-ALL was >4 log in the presence of both human RBC and BM. In patient 7 the residual leukemia was 1.4% (N1/N2). These cells were CD8$^+$, and the incubation with (RFT2 [anti-CD7] + RFT8 [anti-CD8]) cocktail was fully efficient (>4 log kill). In the BM sample of patient 8, five residual TdT+ cells were observed per 5,000 RBC (0.1% of TdT+ blasts, N1/N2). This BM, only partially involved, contained residual HLA-DR+, CD7−, TdT+ cells (normal BM precursors). These were, again, preserved during the treatment with MoAb to CD7 and C′.

It is relevant that in the unpurged samples of patients 2, 6, and 7 morphologically identifiable blast cells (10% to 22%) were TdT− but still expressed the relevant leukemia-associated membrane antigens: CD10 in patient 2 (see arrow in Fig 1A), CD7 in patient 6, and CD7 + CD8 in patient 7. Thus the proportions of residual TdT− blast cells after the purge were low. By contrast, the BM smears from patient 9 had a mixed lymphoid/myeloid blastic transformation of a chronic granulocytic leukemia (Philadelphia chromosome–positive). The two blast cell populations were CD10$^+$, TdT+ lymphoid, and MCS-2+, TdT− myeloid. After having been incubated with RFAL-3 (anti-CD10) and rabbit C′, the purged sample had few TdT+ cells (N1: 66 representing 3.3% of TdT+ leukemia). Nevertheless, 71% leukemic cells, TdT− myeloid blasts, were seen (Fig 2E).

Finally, patient 10 had 88% blasts (L3 morphology, RFB7+, HLA-DR+, IgM+, TdT−). B blasts were eliminated by RFB7 and C′ as was confirmed by the disappearance of IgM+ cells (N1/N2, <0.025%).

DISCUSSION

MoAbs of different isotypes are available against the same leukocyte differentiation antigens (CD clusters).[22,23] Their use with isotype-specific second layers together with the availability of directly labeled and biotin-conjugated Abs facilitates the clinical applications of double-color IF systems.[17,33] The microplate method summarized before is useful for the following reasons: (1) This rapid assay with multiple sample-handing yields results for 12 Abs within less than 1 hour. This first round includes the CD groups of MoAbs that are also available commercially and uniformly used in many laboratories (Table 1).[22,23] (2) The analysis selects relevant membrane-labeled blasts for immediate double labeling with anti-TdT in order to define the immunologic diagnosis in leukemias with low counts and with mixed features (see patients 4 and 9, respectively, Table 3). (3) Finally, following the rapid diagnosis, the C′-mediated lysis with C′-fixing MoAbs and their cocktails is studied as the necessary first step to investigate in individual patients the feasibility of BM purging during autologous BMT.

In order to make these assays quantitative and sensitive, two features have been introduced. First, after the leukemic cells were mixed with known numbers of "bystanders," or inert cells, in roughly equal proportions, their numbers were counted in a unit volume of inert cells (eg, 5,000 cells) before and after C′-mediated lympholysis. In cases with <0.02% residual leukemia, additional inert cells have been scanned in an even larger area of cell spreads ($>2 \times 10^4$) in order to observe >4 log kill. It has been demonstrated before that the simplest inert cells are human RBC, but the C′-mediated lympholysis is also effective in the presence of normal BM (Table 3, patients 3b and 6b). Second, to follow leukemic cells through this process, a robust marker, independent of the Abs used for C′ lysis, was employed. Anti-TdT antibodies[18] are well suited for this purpose as they detect nuclear antigens in immature lymphoid cells and their malignant counterparts. This cellular localization helps the rapid scanning of cytospin spots on slides. We have demonstrated three different membrane marker–TdT combinations that employ MoAbs as primary reagents. Other independent membrane markers such as monoclonal Ig for malignant B cells in patient 10 or cytoplasmic staining with MoAbs to CD22[34] are also suitable.

During the standardization of the purging assay two surprising observations were made. First, it has been shown that the method is suitable to identify even single TdT+ cells on slides carrying $>2 \times 10^4$ inert cells. Thus the study of >4 log kill is feasible. Four examples have demonstrated how to exploit such sensitivity: (1) Patient 8 had originally presented with T-ALL, and after efficient purging using RFT2 MoAb (anti-CD7), the few residual TdT+ cells (0.1%)

expressed the phenotype of normal BM precursors. (2) In patient 2 the assay gave an early indication about the partial loss of C′ activity in the stored rabbit serum, but a new batch completely cleared TdT+ blasts. (3) Our assay was necessary to search for MoAb cocktails that provided an apparently complete lysis of TdT+ leukemia in the presence of human C′ (Janossy, Campana, and Bekassy; manuscript in preparation). (4) Finally, in samples from patients 3 and 7 the single MoAbs used (RFAL-3 and RFT2, respectively) were insufficient to give full lysis with rabbit C′ (1.4% residual TdT+ cells in both). With the help of this assay a more efficient MoAb cocktail was found in patient 7. It can therefore be concluded that the sensitivity of the purging assay is useful.

The other unexpected observation has been that in relatively large proportions of leukemias an effective (>4 log) elimination of TdT+ cALL and T-ALL blast cells could be achieved with rabbit C′ and single MoAbs (48% of ALL cases tested) or with MoAbs cocktails (77% of patients; Janossy and Campana, manuscript in preparation). These MoAbs and C′ are now in use to decrease the malignant contamination in BM samples taken from patients in remission. These observations taken together with the excellent >3 log lysis in three BM samples harvested in relapse indicate that fresh leukemic cells show the same or even increased sensitivity to C′-mediated lysis when compared with cell lines.[12,13]

Our study is in agreement with previous observations that, partly by the help of clonogenic assays obtained with continuously growing cell lines of different (ie, cALL, T, or B) origin, have established the conditions for C′-mediated lysis. These include two rounds of C′ incubation for 45 to 60 minutes each and the use of DNAse.[4,11,12] In addition, we used 30-day rabbit C′ taken from 1-month-old animals with low levels of maternal Ig. This nontoxic C′ source is easy to standardize. In all these studies the cytoreduction of MoAb-reactive cells was mostly >4 log[11,12] although certain cell lines such as Ly67[11] and KM3[12] were relatively resistant with only a 1 to 2 log decrease in spite of good MoAb reactivity. It remains unsolved whether this resistance has been acquired during the selection of cell lines or whether it represents the inherent features of some cases of leukemia/lymphoma.

Most of these clonogenic studies have not been designed to investigate the elimination of unwanted cells in a clinical setting. Only very recently has it become possible to introduce liquid culture assays for freshly established lines of Burkitt origin that still closely reflect the features of the original tumor.[35] With the help of this test it has been possible to demonstrate a >6 log kill with MoAb cocktails of RFB7, SB4, and Y29/55, a cytoreduction that can be achieved with magnetic beads only when this technique is most carefully standardized (M. Favrot and I. Philip, manuscript in preparation). It is more difficult to grow ALL blasts freshly obtained from patients.[36,37] The serum and growth factors are not yet fully characterized, and the plating efficiency is lower than with Burkitt's lymphoma.

In conclusion, the techniques described here will contribute not only to the selection of patients but also to that of the therapeutic MoAbs that fix rabbit and human C′. It is envisaged that our methods will help to find the tailor-made conditions for purging the BM in individual patients including the quality control of the clinical procedure, while the complementary clonogenic assays for ALL, perhaps after further development for reproducibility, may yield additional results for confirming the degree of depletion above the 4 log range of cytoreduction, as has been recently demonstrated in fresh explants obtained from Burkitt's lymphomas.[35]

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REFERENCES

1. Prentice HG, Blacklock HA, Janossy G, Gilmore MJML, Price-Jones L, Tidman N, Trejdosiewicz LK, Skeggs DBL, Panjwani D, Ball S, Graphakos S, Patterson J, Ivory K, Hoffbrand AV: Depletion of T lymphocytes in donor marrow prevents significant graft versus host disease in matched allogeneic leukaemic marrow transplant recipients. Lancet i:472, 1984

2. Waldmann H, Polliak A, Hale G, Or R, Cividelli G, Weiss L, Wechsler Z, Samuel S, Manor D, Brauthar C, Rachmilewitz EA, Slavin S: Elimination of graft-versus host disease by in vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). Lancet 2:483, 1984

3. Filipovich AH, Vallera D, Youle RJ, Quinones RR, Neville DM, Kersey JH: Ex-vivo treatment of donor bone marrow with anti-T cell immunotoxins for prevention of graft-versus-host disease. Lancet 1:469, 1984

4. Janossy G, Prentice HG, Grob J-P, Ivory K, Tidman N, Grundy J, Favrot M, Brenner M, Campana D, Blacklock HA, Gilmore MJML, Patterson J, Griffiths P, Hoffbrand AV: T lymphocyte regeneration after transplantation of T cell depleted allogeneic bone marrow. Clin Exp Immunol 63:577, 1986

5. Bast RC, Ritz J, Lipton JM, Feeney M, Sallan SE, Nathan DG, Schlossman SF: Elimination of leukaemic cells from human bone marrow using monoclonal antibody and complement. Cancer Res 43:1389, 1983

6. Ramsay N, LeBien T, Nesbit M, McGlave P, Weisdorf D, Kenyon P, Hurd D, Goldman A, Kim T, Kersey J: Autologous bone marrow transplantation for patients with acute lymphoblastic leukemia in second or subsequent remission: Results of bone marrow treated with monoclonal antibodies BA-1, BA-2, BA-3 plus complement. Blood 66:508, 1985

7. Nadler LM, Botnick L, Finberg R, Canellos GP, Takvorian T, Bast RC, Hellman S, Schlossman ST: Anti-B1 monoclonal antibody and complement treatment in autologous bone marrow transplantation for relapsed B-cell non-Hodgkin's lymphoma. Lancet 2:427, 1984

8. Baumgartner C, Bleher EA, Brun del Re G, Bucher U, Deubelbeiss KA, Greiner R, Hirt A, Imbach P, Luthy A, Odavic R, et al: Autologous bone marrow transplantation in the treatment of children and adolescents with advanced malignant tumors. Med Pediatr Oncol 12:104, 1984

9. Van Dongen JJM, Hooijkaas H, Hahlen K, Benne K, Bitter WM, van der Linde-Preesman AA, Tettero ILM, van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A: Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining, in Lowenberg P, Hagenbeck J (eds): Minimal Residual Disease in Acute Leukaemia. The Hague, Nijhoff, 1984, p 67

10. Bast RG, De Fabritiis P, Lipton J, Gelber R, Maver C, Nadler L, Sallan S, Ritz J: Elimination of malignant clonogenic cells from human bone marrow using multiple monoclonal antibodies and complement. Cancer Res 45:499, 1985

11. Favrot MC, Philip I, Philip T, Pinkerton R, Lebacq AM, Forster K, Adeline P, Dore JF: Bone marrow purging procedure in Burkitt lymphoma with monoclonal antibodies and complement. Quantification by a liquid cell culture monitoring system. Br J Haematol 1986 (in press)

12. LeBien TW, Stepan DE, Bartholomew RM, Strong RC, Anderson JM: Utilization of a colony assay to assess the variable influencing elimination of leukemic cells from human bone marrow with monoclonal antibodies and complement. Blood 65:945, 1985

13. Myers CD, Thorpe PE, Ross WCJ, Cumber AJ, Katz FE, Greaves MF: An immunotoxin with therapeutic potential in T-cell leukemia: WT1-ricin A. Blood 63:1178, 1984

14. Casellas P, Canat X, Fauser AA, Gros O, Laurant G, Poncelet P, Jansen FK: Optimal elimination of leukemic T cells from human bone marrow with T101-ricin A-chain immunotoxin. Blood 65:289, 1985

15. Hagemeijer A, Adriaansen HJ: New possibilities for cytogenetic analysis, in Lowenberg P, Hagenbeck J (eds): Detection and Treatment of Minimal Residual Disease in Acute Leukemia. The Hague, Nijhoff, 1986 (in press)

16. Bollum FJ: Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 54:1203, 1979

17. Janossy G, Campana D, Bollum FJ: Immunofluorescence studies in leukaemia diagnosis, in Beverley P (ed): Monoclonal Antibodies. Methods in Haematology. New York, Churchill Livingstone, 1985, p 97

18. Bollum FJ, Augl C, Chang LMS: Monoclonal antibodies to human terminal transferase. J Biol Chem 259:5848, 1984

19. Johnson GD, Holborow EJ: Immunofluorescence, in Weir DM, Herzenberg LA (eds): Handbook of Experimental Immunology (ed 4). London, Blackwell, 1985, p 317

20. Tindle RW, Nichols RAB, Chan L, Campana D, Catovsky D, Birnie GT: A new monoclonal antibody B1-3C5 recognizes myeloblasts and non-T, non-B lymphoblasts in acute leukaemia and chronic granulocytic leukaemia blast crisis. Leuk Res 9:1, 1985

21. Linch DC, Allen C, Beverley PCL, Bynoe AG, Scott CS, Hogg N: Monoclonal antibodies differentiating between monocytic and non-monocytic variants of AML. Blood 63:566, 1984

22. Reinherz EL, Haynes BF, Nadler LM, Bernstein ID: Human B Lymphocytes. Leucocyte Typing II, vol 2. New York, Springer-Verlag, 1986

23. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF: Leucocyte Typing I. New York, Springer-Verlag, 1984

24. Batory G, Bofill M, Petranyi GS, Janossy G, Hollan SR: Comparative analysis of monoclonal antibodies of workshop T series on suspensions and tissue sections, in Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF (eds): Leucocyte Typing. New York, Springer-Verlag, 1984, p 469

25. Guesdon JL, Ternyuck T, Avrameas S: The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem 27:1131, 1979

26. Janossy G, Bofill M, Poulter LW: Two colour immunofluorescence: Analysis of the lymphoid system with monoclonal antibodies, in van Noorden S, Polak J (eds): Immunocytochemistry Today. Oxford, Wright, 1985

27. Sanofi Monoclonal Antibodies for Cell Labelling: Sanofi Recherche, Montpellier, France, 1986

28. Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8:521, 1984

29. Drexler HG, Sagawa K, Menon M, Minowada J: Reactivity pattern of myeloid monoclonal antibodies with emphasis on MCS-2. Leuk Res 10:17, 1986

30. Kersey JH, Goldman A, Abramson C, Nesbit M, Perry G, Gajl-Peczalska K, Le Bien T: Clinical usefulness of monoclonal antibody phenotyping in childhood acute lymphoblastic leukaemia. Lancet 2:1419, 1982

31. Janossy G, Bollum FJ, Bradstock KF, McMichael A, Rapson N, Greaves MF: Terminal transferase positive human bone marrow cells exhibit the antigenic phenotype of common acute lymphoblastic leukemia. J Immunol 123:1525, 1979

32. Bradstock KF, Janossy G, Hoffbrand AV, Ganeshaguru K, Llewelin P, Prentice HG, Bollum FJ: Immunofluorescent and biochemical studies of terminal deoxynucleotidyl transferase in treated acute leukaemia. Br J Haematol 47:121, 1981

33. Lanier LL, Phillips JH, Warner NL: Monoclonal antibodies to human lymphocytes, in Beverley PLC (ed): Monoclonal Antibodies. Methods in Haematology. New York, Churchill Livingstone, 1985, p 207

34. Campana D, Janossy G, Bofill M, Trejdosiewicz LK, Ma D, Hoffbrand AV, Mason DY, Lebacq A-M, Forster H: Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 134:1524, 1985

35. Philip I, Favrot MC, Philip T: Use of a liquid cell culture assay to quantify the elimination of Burkitt lymphoma cells from the bone marrow. J Immunol Methods 1986 (in press)

36. Touw I, Delwel R, Bolhuis R, van Zanen G, Lowenberg B: Common and pre-B acute lymphoblastic leukemia cells express interleukin 2 receptors, and interleukin 2 stimulates in vitro colony formation. Blood 66:556, 1985

37. Uckun FM, Gajl-Peczalska KJ, Kersey JH, Houston LL, Vallera DA: Use of a novel colony assay to evaluate the cytotoxicity of an immunotoxin containing pokeweed antiviral protein against blast progenitor cells freshly obtained from patients with common B-lineage acute lymphoblastic leukemia. J Exp Med 163:347, 1986

Biosynthesis of Factor XIII B Subunit by Human Hepatoma Cell Lines

By Janice A. Nagy, Per Henriksson, and Jan McDonagh

The plasma transglutaminase, factor XIIIa (FXIIIa), circulates as a zymogen containing two proteins, A and B, arranged in a noncovalent tetrameric complex, A_2B_2. Biosynthesis of plasma FXIII has not previously been demonstrated. In the present study, direct evidence has been obtained that two human hepatoma cell lines, Hep G2 and PLC/PRF/5, synthesize and secrete FXIII B protein. Secretion of the B subunit of FXIII by Hep G2 was demonstrated by immunoblotting. De novo synthesis by Hep G2 was confirmed in ^{35}S-methionine–labeled cultures. Radiolabeled conditioned medium was concentrated, mixed (1:1) with purified B protein, and examined by crossed immunoelectrophoresis with antiserum to the B subunit. The single protein precipitin arc of purified B protein comigrated with the radiolabeled FXIII from Hep G2 visualized by autoradiography, indicating both electrophoretic and antigenic identity. The data presented here represent the first demonstrations of biosynthesis of FXIII B protein by any cell type and suggest that the liver is the site of synthesis of FXIII B protein. Further analysis of concentrated Hep G2 serum-free conditioned medium (SFCM) and cell lysate by immunoblotting following nondenaturing agarose gel electrophoresis demonstrated the FXIII A protein as well as the B protein and also revealed synthesis and secretion of the A and B proteins by PLC/PRF/5. Crossed immunoelectrophoresis studies of Hep G2 SFCM and cell lysate suggest that Hep G2 cells also synthesize and secrete the plasma FXIII zymogen. With a specific radioimmunoassay for B protein, FXIII was found in Hep G2 SFCM at approximately 4 ng/mL; with an amplified rocket immunoelectrophoresis technique the level was ~5 ng/mL.

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FACTOR XIII (FXIII) is a zymogen of the blood coagulation system that in its active form, functions as a transglutaminase to stabilize fibrin clots by catalyzing the formation of ϵ-(γ-glutamyl)-lysyl cross-links between the γ chains and between the α chains of fibrin monomers.[1,2] FXIII is essential for normal hemostasis, and it may also have a more general role in various processes involving cell proliferation, including wound healing and tissue repair, tumor growth and metastasis, and atherosclerosis.[3] The FXIII proenzyme is found as both an extracellular and an intracellular zymogen.[4] In plasma, FXIII is composed of two nonidentical polypeptide chains, A (molecular weight [mol wt], 75,000) and B (mol wt, 87,000), that are associated noncovalently as a tetrameric complex with the molecular formula A_2B_2.[5,6] Intracellularly, the FXIII zymogen consists of only the A subunit in the dimeric form A_2.[5] The transglutaminase activity resides in the A subunit and is dependent on an active-site sulfhydryl group.[6,7] Generation of full enzymatic activity from either form of the zymogen requires proteolytic modification of the A subunit by thrombin and conformational alteration of the A subunit by calcium ions.[7,8] In the case of plasma FXIII, full activation is also accompanied by dissociation of the B subunit. In addition to its distribution in plasma and platelets, FXIII has been found in placenta, uterus, megakaryocytes, and prostate tissue.[9-11] More recently, FXIII has been demonstrated in monocytes and peritoneal macrophages.[12-14] All of these intracellular zymogens consist of only A subunits, and they are immunochemically and electrophoretically identical to the platelet zymogen.[9-14]

Although FXIII has been demonstrated in various tissue types, the actual site or sites of synthesis have not been unequivocally determined. Strong but indirect evidence indicates that the megakaryocyte, the platelet progenitor, is the synthetic site for platelet FXIII.[9] The human monocyte-macrophage cell line U937 has been shown to synthesize A protein,[12] but synthesis of intracellular FXIII in other tissues has not been reported. Little is known concerning the biosynthesis of plasma FXIII. The liver has been suggested as the site of synthesis, since some patients with chronic liver disease have decreased levels of FXIII activity in plasma.[1,15] However, other studies have shown that plasma FXIII levels can remain normal in most cases of hepatic disease.[1] Immunofluorescent and immunoperoxidase techniques have also been used to search for an intracellular location of plasma FXIII, with conflicting results. Two such studies using hepatocytes indicated that FXIII A and B proteins are located in the liver[16,17]; however, another study reported the presence of both FXIII A and B subunits in placenta, megakaryocytes, and in fibroblasts but found hepatocytes to be negative for both A and B proteins.[18]

The study presented here was undertaken to address the question of the site of synthesis of plasma FXIII. A well-characterized human hepatoma cell line, Hep G2, has morphological characteristics similar to those of normal liver parenchymal cells[19] and has the ability to secrete a number of human plasma proteins,[20] including those involved in fibrinolysis,[21,22] coagulation,[23-27] and complement activation.[28,29] In this study, Hep G2 was selected as a prototype of hepatocytes and used to investigate FXIII biosynthesis. These cells have been found to synthesize and release the B protein into the culture medium. The secreted FXIII B protein is immunochemically identical to the plasma FXIII B subunit. In addition, the synthesis and secretion of the B protein by another human hepatoma cell line, PLC/PRF/5, has been documented. This work provides firm biochemical evidence to suggest that the liver is the site of biosynthesis of plasma

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FXIII B protein. Furthermore, preliminary studies suggest that Hep G2 and PLC/PRF/5 cells also synthesize the FXIII A protein and synthesize a FXIII zymogen identical to extracellular or plasma FXIII (A_2B_2).

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), methionine-free minimum essential medium (MEM), trypsin-EDTA, Hanks' balanced salt solution (HBSS), and penicillin/streptomycin solutions were obtained from GIBCO Laboratories (Grand Island, NY); fetal bovine serum (FBS) was from Whittaker M.A. Bioproducts (Walkersville, MD). L-[^{35}S]-methionine (1,400 Ci/mmol) and [1,4(*n*)-^{3}H] putrescine (15 Ci/mmol) were purchased from Amersham Corp (Arlington Heights, IL), and ^{125}I-protein A from Dupont NEN Research Products (Wilmington, DE). Immunoelectrophoresis buffer I (0.1 mol/L barbital), acrylamide, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Richmond, CA), SeaKem Agarose ME and Gel Bond film NF were from FMC Marine Colloids, (Rockland, ME). Nitrocellulose sheets (0.22 µmol/L) were purchased from Schleicher and Schuell (Keene, NH), XAR-2 film from Eastman Kodak Co. (Rochester, NY), and Ultrafilm from LKB (Gaithersburg, MD). Centriflo CF25 ultrafiltration membrane cones were obtained from Amicon Corp (Danvers, MA) and Pansorbin from Calbiochem-Behring Corp (La Jolla, CA). Prestained protein high–mol wt standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD) and bovine thrombin from Parke-Davis Co (Morris Plains, NJ). Phenylymethylsulfonylfluoride (PMSF), *p*-chloromercuribenzoate, ovalbumin, and benzamidine were obtained from Sigma Chemical Co (St Louis).

Plasma FXIII was prepared from recovered human plasma (American Red Cross Blood Program, northeast region) as previously described.[30] Purified FXIII B subunit was obtained from the thrombin-treated zymogen by affinity chromatography on organomercurial agarose.[31] Purified FXIII A_2 was prepared from placental concentrate (Fibrogammin, Behringwerke AG, Marburg, Germany, kindly provided by Drs Heimburger and Karges) as described.[32] The rabbit antiserum to the B subunit of FXIII has been previously described.[33] The rabbit antiserum to the A subunit of FXIII was purchased from Calbiochem-Behring (Clotimmune anti-FXIII A). ^{125}I-FXIII A_2 and ^{125}I-FXIII A_2B_2 were prepared using Na ^{125}I (Dupont NEN Research Products) and Enzymobeads (Bio-Rad) by the solid-phase lactoperoxidase–glucose oxidase procedure.[34] After labeling, each radioligand was repurified on diethyl aminoethyl–high-performance liquid chromatography with linear salt gradient solution.

Cell culture. The human hepatoma cell line Hep G2 was provided by Drs Barbara B. Knowles and David P. Aden of the Wistar Institute, Philadelphia. PLC/PRF/5 is a tissue culture cell line derived from a human hepatocellular carcinoma established by Alexander et al[35] and was provided by Dr I.M. Arias of Tufts New England Medical Center. Both cell lines were cultured in DMEM supplemented with 10% FBS, pencillin (100 U/mL), and streptomycin (100 µg/mL). To prepare large quantities of serum-free conditioned media (SFCM), cells were seeded in T175 flasks at a density of 1.5 × 10^7 cells/flask in 50 mL of medium. When the cells had become adherent but not confluent (usually within one to two days), cultures were washed three times with 10 mL of serum-free medium and then incubated in 50 mL of serum-free medium for 72 hours. The SFCM was removed, clarified by centrifugation, concentrated 100-fold with Amicon Centriflo CF25 ultrafiltration membrane cones, and stored at 4 °C or at −80 °C.

Cell lysate was prepared from confluent cultures. The cells were dissociated by trypsinization, washed three times in HBSS, and lysed at a density of 10^8 cells/mL by the addition of 1% Triton X-100, 10 mmol/L Tris-HCl, 100 mmol/L NaCl, 1 mmol/L EDTA, pH 7.5, for 60 minutes at 4 °C. After centrifugation for 30 minutes at 100,000 g, the supernatant was removed and assayed immediately for FXIII.

Immunoprecipitation and immunoblotting of FXIII. Hep G2 SFCM concentrated 100-fold (250 µL) was added to anti-FXIII B antiserum (10 µL) and incubated for 18 hours at 4 °C. Fifty microliters of Pansorbin was added, and the immunoprecipitates were collected by centrifugation, washed in buffer (0.01 mol/L Tris HCl, 0.1% Triton X-100, 0.5% nonidet P40 (NP-40), 0.5% deoxycholate (DOC) 0.1% SDS, 0.05% Tween 20, 2 mmol/L EDTA, 1 mmol/L PMSF, 0.14 mol/L NaCl, pH 8.0), and solubilized by boiling for ten minutes in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (10% glycerol, 3% SDS, 0.025 mol/L Tris-HCl, pH 6.8, 0.01% bromophenol blue, 5% 2-mercaptoethanol). Purified FXIII A_2B_2, A_2 or B subunit and the dissolved immunoprecipitates from Hep G2 SFCM were electrophoresed[36] and then electrophoretically transferred to nitrocellulose.[37] After the transfer, the nitrocellulose was washed with Blotto[38] for 30 min, followed by 3% hemoglobin 0.01 mol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.3, and 0.1% sodium azide (Hb/PBSa, Sigma, St Louis) for 30 min then reacted with monospecific antiserum to FXIII B subunit diluted in 3% Hb/PBSa. Detection of FXIII B subunit was facilitated by the addition of ^{125}I-protein A followed by autoradiography.

Metabolic labeling of intracellular and secreted proteins. Confluent cultures of Hep G2 cells (5 × 10^7 cells/T175 flask) were rinsed three times with labeling medium and incubated with 15 mL of labeling medium for 15 minutes at 37 °C, 5% CO_2. The labeling medium prepared from the MEM Selectamine Kit (GIBCO) lacked FBS and contained 10 µmol/L rather than 100 µmol/L L-methionine. L-[^{35}S]-methionine was added to a concentration of 100 to 150 µCi/mL, and the cells were incubated at 37 °C, 5% CO_2 for five hours. The conditioned medium containing the intrinsically labeled proteins secreted by the Hep G2 cells was recovered, clarified by centrifugation, and concentrated 100-fold by ultrafiltration. The cells were dissociated by trypsinization, washed three times in PBS and lysed by the addition of lysis buffer (1% Tritox X-100, 0.5% DOC in PBS, 4 °C, one hour). After centrifugation for 30 minutes at 100,000 g, the supernatant was concentrated 20-fold by ultrafiltration. The concentrated lysate and conditioned medium were both stored at −80 °C.

Crossed immunoelectrophoresis (CIE). CIE was performed in 0.8% agarose in 0.1 mol/L barbital buffer and 2 mmol/L EDTA, pH 8.6.[39,40] Concentrated conditioned medium or the cell extract of L-[^{35}S]-methionine–labeled cultures of Hep G2 was mixed 1:1 with either purified B protein, normal plasma, or purified FXIII A_2B_2. Electrophoresis in the first dimension was carried out at 10 °C at 10 to 12 V/cm for two hours. Strips of agarose were then transferred to an 0.8% agarose gel containing 0.5% antiserum to the B subunit. Electrophoresis was carried out in the second dimension for 18 hours at 2 V/cm, 10 °C. Gels were then washed in saline, rinsed, covered with filter paper, compressed, and rehydrated with saline. This washing procedure was repeated three times to remove residual, nonimmunoprecipitated proteins. Gels were then dried onto Gel Bond film and stained with Coomassie brilliant blue. The CIE gels were then subjected to autoradiography with LKB Ultrafilm.

Agarose gel electrophoresis. Agarose gel electrophoresis was performed at 10 °C and 20 V/cm in 1-mm-thick gels containing 0.8% agarose for 1.5 hours in 0.1 mol/L barbital buffer and 2 mmol/L EDTA, pH 8.6.[41] The proteins were transferred from the agarose gel to nitrocellulose by capillary action.[42] The nitrocellulose was then treated as described above with either anti-FXIII A or B antiserum followed by ^{125}I-protein A.

Rocket immunoelectrophoresis. Quantitation of the FXIII in Hep G2 SFCM was performed immunochemically by rocket immunoelectrophoresis[43] coupled with an amplification procedure developed specifically for the detection of nanogram quantities of FXIII A_2B_2 antigen. Electrophoresis was carried out in agarose (1% wt/vol) containing anti-FXIII B antiserum in Tris-barbital buffer (0.03 mol/L Tris, 0.1 mol/L barbital, pH 8.6) at 5 V/cm, 10 °C for 18 hours. After electrophoresis, the gel was incubated with 50 mL of ^{125}I–protein A in 3% Hb/PBSa (150,000 cpm/mL) for one hour, covered with filter paper, and compressed with a weight. The gel was then rehydrated and washed in saline. This procedure was repeated three times to ensure the complete removal of an ^{125}I–protein A not bound to the precipitate. The gel was dried onto a sheet of Gel Bond film and subjected to autoradiography with XAR film. Pooled human plasma, used as a standard, was assumed to contain 30 µg/mL FXIII.[32] The precision of this method was ±5% in the height of the rocket, and the sensitivity was 0.5 ng of FXIII A_2B_2.

RESULTS

SFCM from Hep G2 cell cultures was examined for the presence of FXIII B protein by means of immunoblotting. Initially, Hep G2 SFCM was treated with Blue Sepharose (Pharmacia, Piscataway, NJ) to remove cell-secreted albumin (which caused severe distortion of the protein band in the area of the FXIII subunit) and then concentrated by ultrafiltration. By SDS-PAGE followed by immunoblotting, the B protein was detected as a very faint band in the autoradiogram, and the mobility of the subunit was similar to that of the purified polypeptide chain. However, despite removal of most of the interfering albumin, the amount of Hep G2 SFCM that could be loaded into one well of the polyacrylamide gel without band distortion limited the level of detection of FXIII B protein by this method.

To overcome these limitations, immunoprecipitation was coupled with the immunoblotting detection system. Monospecific anti-FXIII B antiserum was used to immunoprecipitate FXIII from the concentrated Hep G2 SFCM. The immunoprecipitate was then electrophoresed on SDS–polyacrylamide gels, transferred to nitrocellulose, and detected with anti-FXIII B antiserum. Figure 1 shows the results of such an experiment. Lane 2 contains the immunoprecipitated material from the concentrated (100-fold) Hep G2 SFCM. Included for comparison in the gel are FXIII A_2B_2 (lane 1), FXIII A_2 (lane 3), and FXIII B protein (lane 4). These results shows that the anti-FXIII B antiserum did not cross-react with the A subunit and that the mobility of the band in the immunoprecipitated material from the Hep G2 SFCM that is recognized by the anti-FXIII B antiserum is identical to that of the B subunit of FXIII. The intensity of the FXIII B subunit detected in the Hep G2 SFCM was dependent on the amount of SFCM used for the immunoprecipitation as well as the degree to which the Hep G2 SFCM has been concentrated. This method provided a means of further concentrating the FXIII B protein from the SFCM (already concentrated 100-fold) prior to electrophoresis.

Direct evidence that the FXIII detected in Hep G2 SFCM was specifically synthesized and secreted by these cells was obtained by metabolic protein labeling with L-[^{35}S]-methionine. Demonstration that the intrirsically labeled FXIII was identical to FXIII B protein was obtained by CIE. Radiolabeled conditioned medium was concentrated 100-fold, mixed with purified FXIII B protein, and anayzed by CIE in agarose gels that contained antiserum to the FXIII B subunit in the second dimension. The protein-stained pattern is shown in Fig 2A and the corresponding autoradiograms in Fig 2B. The Coomassie staining pattern and the corresponding autoradiogram showed single immunoprecipitation arcs with identical mobility and area. Figure 2 shows that the radiolabeled FXIII present in the culture medium comigrated with purified FXIII B protein, indicating both electrophoretic and antigenic identity. These results imply that the intrinsically labeled FXIII was identical to the FXIII B protein.

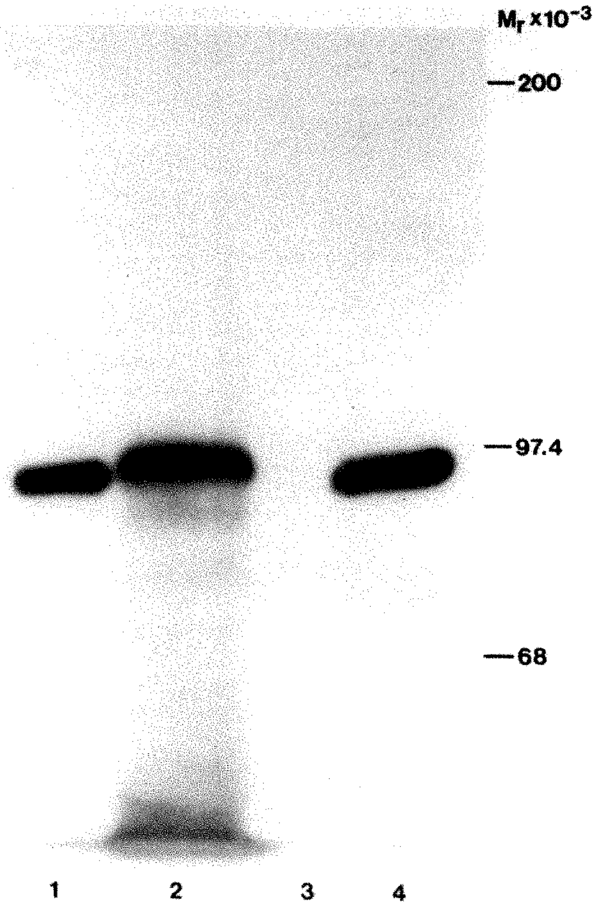

Fig 1. Identification of FXIII B subunit secreted by Hep G2. Immunoblot of purified and Hep G2 FXIII analyzed by SDS-PAGE (7% resolving, 3% stacking) under reducing conditions. The proteins were transferred to nitrocellulose electrophoretically (70 V, 600 mA, 10 °C, 18 hours). The nitrocellulose paper was incubated with antiserum to FXIII B subunit, and the reacting proteins were detected by reaction with ^{125}I–protein A followed by autoradiography. Lane 1, 140 ng of purified FXIII A_2B_2; lane 2, immunoprecipitate from 250 µL of 100-fold–concentrated SFCM and 10 µL of anti-FXIII B subunit antiserum; lane 3, 120 ng of purified FXIII A_2; lane 4, 140 ng of purified FXIII B subunit. Mol wt standards were myosin, 200,000; phosphorylase B, 92,500; BSA, 68,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; lactalbumin, 18,400; and cytochrome C, 12,300.

Efforts to immunoprecipitate the FXIII A protein from the Hep G2 SFCM with anti-FXIII A antiserum and to

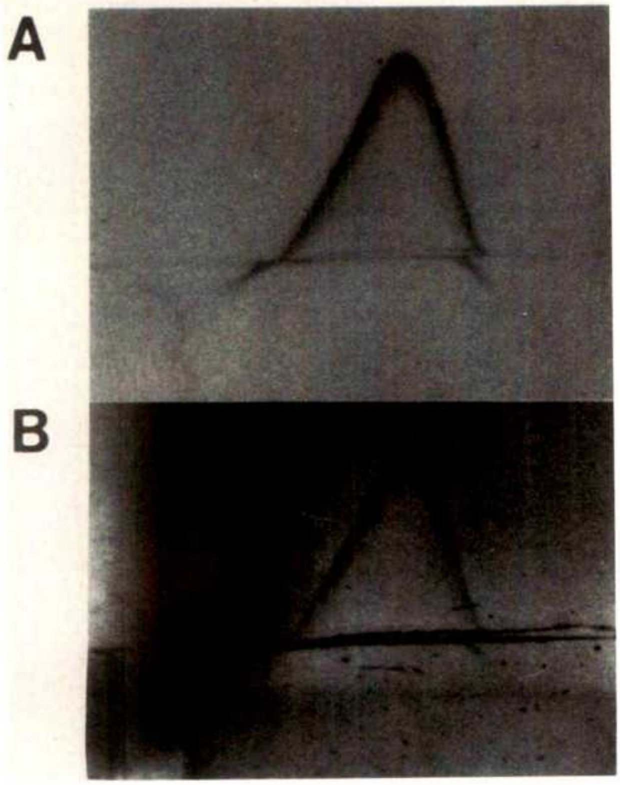

Fig 2. Analysis of the FXIII protein synthesized and secreted by Hep G2 by CIE. Cultures of Hep G2 cells were intrinsically labeled with L-[^{35}S]-methionine. The supernatant from these cells was concentrated and added to purified FXIII B protein and electrophoresed *v* agarose containing anti-FXIII B antiserum at a concentration of 0.5%. Panel A contains the Coomassie blue-stained immunoprecipitate while panel B contains the corresponding autoradiogram. Fifteen microliters of 100-fold-concentrated Hep G2 SFCM were used plus 15 μL of FXIII B protein, 30 μg/mL.

detect it following SDS-PAGE by immunoblotting or to detect it by CIE with anti-FXIII A antiserum were largely unsuccessful. In addition, when FXIII in Hep G2 SFCM was immunoprecipitated with anti-FXIII B antiserum, no FXIII A protein was detected by immunoblotting using anti-FXIII A antiserum in an SDS-polyacrylamide gel system. However, the FXIII A subunit was detected (in a degraded form in SFCM) by nondenaturing agarose gel electrophoresis coupled with immunoblotting.

The technique of agarose gel electrophoresis under nondenaturing conditions has previously been used to study the FXIII proteins.[44] Figures 3 and 4 show the electrophoretic pattern of various molecular forms of FXIII compared with the FXIII found in Hep G2 SFCM. Detection of FXIII B with anti-FXIII B antiserum is shown in Fig 3, while detection of FXIII A protein with anti-FXIII A antiserum is shown in Fig 4. In both Figs, lane 1 contained FXIII A_2; lane 2, FXIII A_2B_2; and lane 3, FXIII B protein. Under the nondenaturing conditions used, FXIII A_2B_2 and FXIII A_2 have the same electrophoretic mobility[44] but can be distinguished when this method is coupled with immunoblotting (ie, FXIII A_2 does not react with antiserum to B protein, Fig 3, lane 1, while FXIII A_2B_2 does react, Fig 3, lane 2). The results for the control samples in lanes 1 to 3 from both Figs 3 and 4 indicate that in this system both antisera are specific for their respective FXIII subunits and do not cross-react with the other subunit. Furthermore, the antisera each react with the purified subunit as well as with the subunit in the complexed form. In Figs 3 and 4, lanes 4 and 5 contain concentrated Hep G2 SFCM (100- and 50-fold, respec-

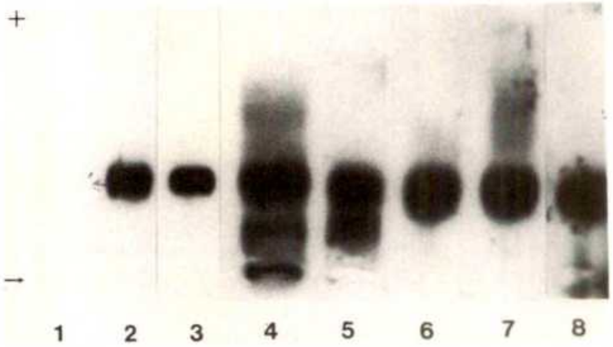

Fig 3. Nondenaturing agarose electrophoresis of purified FXIII proteins and Hep G2 SFCM at pH 8.6: detection of FXIII B subunit. The following samples were used: (1) FXIII A_2, 0.35 μg/mL; (2) FXIII A_2B_2, 0.35 μg/mL; (3) FXIII B, 0.38 μg/mL; (4) Hep G2 100-fold-concentrated SFCM; (5) Hep G2 50-fold-concentrated SFCM; (6) PLC/PRF/5 100-fold-concentrated five-day SFCM; (7) PLC/PRF/5 136-fold-concentrated three-day SFCM; and (8) ^{125}I-labeled FXIII B protein. Twenty microliters of each sample were loaded per slot. After electrophoresis (10 V/cm, 10 °C, two hours), samples were transferred from the agarose gel to nitrocellulose paper by capillary action. The nitrocellulose was treated with anti-FXIII B antiserum followed by ^{125}I-protein A. The bands were visualized by autoradiography. The arrow indicates the sample application well. The direction of migration of FXIII protein is toward the anode.

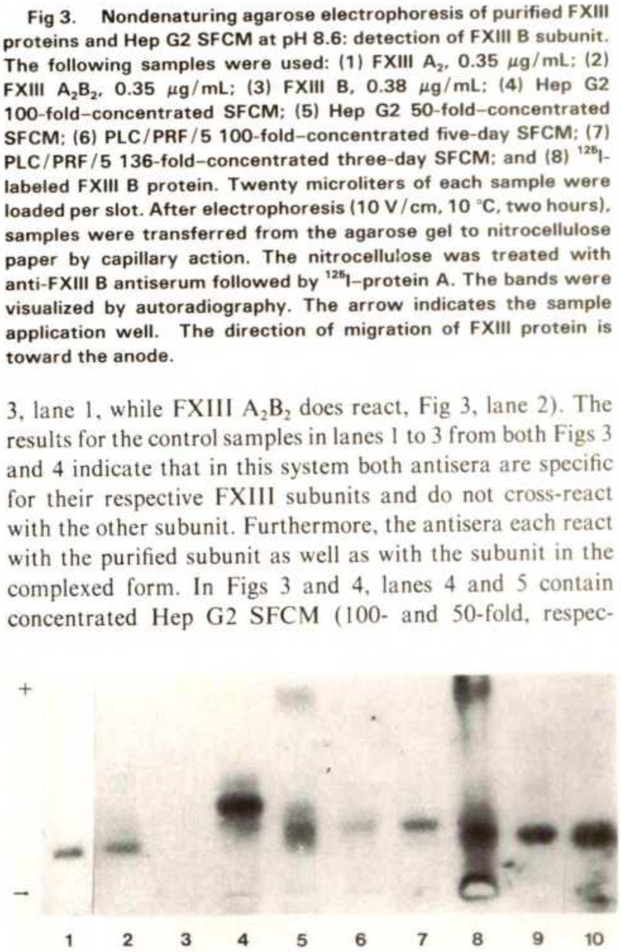

Fig 4. Nondenaturing agarose electrophoresis of purified FXIII proteins and Hep G2 SFCM at pH 8.6: detection of FXIII A subunit. The following samples were used: (1) FXIII A_2, 0.35 μg/mL; (2) FXIII A_2B_2, 0.35 μg/mL; (3) FXIII B, 0.38 μg/mL; (4) Hep G2 100-fold-concentrated SFCM; (5) Hep G2 50-fold-concentrated SFCM; (6) PLC/PRF/5 100-fold-concentrated five-day SFCM; (7) PLC/PRF/5 136-fold-concentrated three-day SFCM; (8) PLC/PRF/5 cell lysate (10^8 cells/mL); (9) Hep G2 cell lysate (10^8 cells/mL); and (10) ^{125}I-labeled FXIII A_2. Twenty microliters of each sample were loaded per slot. After electrophoresis (10 V/cm, 10 °C, two hours) the samples were transferred from the agarose gel to nitrocellulose paper by capillary action. The nitrocellulose was treated with anti-FXIII A antiserum followed by ^{125}I-protein A. The bands were visualized by autoradiography. The arrow indicates the sample application well. The direction of migration of FXIII protein is toward the anode.

tively). These results in Fig 3 indicate that both samples of the Hep G2 SFCM were positive for B protein. The freshly prepared Hep G2 SFCM showed a diffuse band for A protein (Fig 4, lane 5) but the Hep G2 SFCM that had been stored for several weeks at 4 °C showed a band of faster mobility, probably indicating degraded A protein (Fig 4, lane 4).

Concentrated SFCM from another human hepatoma cell line, PLC/PRF/5, was also examined in this agarose gel electrophoresis system. These cells were maintained in culture in the absence of FBS for up to five days with good viability. Figure 3, lanes 6 and 7, shows that two samples of PLC/PRF/5 SFCM were positive for FXIII B protein. In Fig 3, lane 8 shows the mobility of ^{125}I-FXIII B protein. The PLC/PRF/5 SFCM was also positive for FXIII A protein (Fig 4, lanes 6 and 7). The fresh lysates of both cell lines were also found to be positive for A protein (Fig 4, lanes 8 and 9). In Fig 4, lane 10 shows ^{125}I-FXIII A_2 protein. The cell lysates of both lines also showed weak bands for the B protein (data not shown).

Evidence that intrinsically labeled Hep G2 SFCM and cell lysate contained FXIII identical to plasma FXIII in addition to FXIII B protein was obtained by CIE. Radiolabeled conditioned medium and cell layer extracts were concentrated 100-fold and 20-fold respectively, mixed with pooled human plasma or purified FXIII A_2B_2, and analyzed by CIE in agarose gels that contained antiserum to the FXIII B subunit in the second dimension. Protein-stained patterns are shown in Fig 5A and their corresponding autoradiograms in Fig 5B. Lane 1 contains Hep G2 SFCM plus normal plasma, lane 2 contains Hep G2 SFCM added to purified FXIII A_2B_2, and lane 3 shows Hep G2 cell lysate mixed with purified FXIII A_2B_2. The antiserum used in this analysis reacted with normal human plasma to form a single symmetrical peak. In plasma, FXIII is present as the A_2B_2 tetramer; no free B protein has been detected.[33] In each case, the Coomassie staining pattern and the corresponding autoradiogram showed single immunoprecipitation arcs with identical mobility and area. Figure 5 shows that the intrinsically radiolabeled FXIII present in the culture medium or extracted by Triton X-100 from the cell layer comigrated with the FXIII in human plasma or with purified FXIII A_2B_2, indicating both electrophoretic and antigenic identity. These results suggest that this intrinsically labeled FXIII was identical to the plasma FXIII molecule.

Quantification of the FXIII in Hep G2 SFCM was facilitated by development of an amplified rocket immunoelectrophoresis procedure in which trace amounts (not detectable by Coomassie staining) of antigen–antibody immunoprecipitates in agarose are reacted with ^{125}I-protein A and visualized by autoradiography. Concentrated (100-fold) Hep G2 SFCM was electrophoresed into a gel containing anti-FXIII B antiserum (Fig 6). Pooled human plasma was used to determine a standard curve of the height of the rocket *v* concentration of FXIII. The lower limit of detection of FXIII in normal plasma occurs at a 1:512 dilution, which corresponds to a lower limit of detection of 0.5 ng of FXIII A_2B_2 antigen. From the height of the rocket for the Hep G2 SFCM, the level of FXIII antigen in Hep G2 SFCM (unconcentrated) was determined to be approximately 5 to 10 ng/mL. SFCM from Hep G2 cell cultures was also examined for the presence of FXIII A and B subunits by

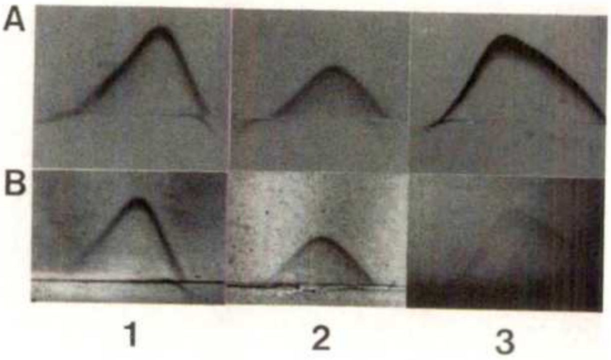

Fig 5. Analysis of FXIII synthesized and secreted by Hep G2 by CIE. Cultures of Hep G2 cells were intrinsically labeled with L-[^{35}S]-methionine. The supernatant or lysate from these cells was concentrated and added to normal plasma or purified FXIII A_2B_2 and electrophoresed *v* agarose-containing anti-FXIII B antiserum at a concentration of 0.5%. Panel A contains the Coomassie blue-stained immunoprecipitates while panel B contains the corresponding autoradiograms. Lane 1, 15 µL of 100-fold-concentrated Hep G2 SFCM plus 15 µL of FXIII A_2B_2, 35 µg/mL; lane 2, 15 µL of 100-fold-concentrated Hep G2 SFCM plus 15 µL of normal plasma; and lane 3, 30 µL of 20-fold-concentrated Hep G2 lysate plus 5 µL of FXIII A_2B_2, 200 µg/mL.

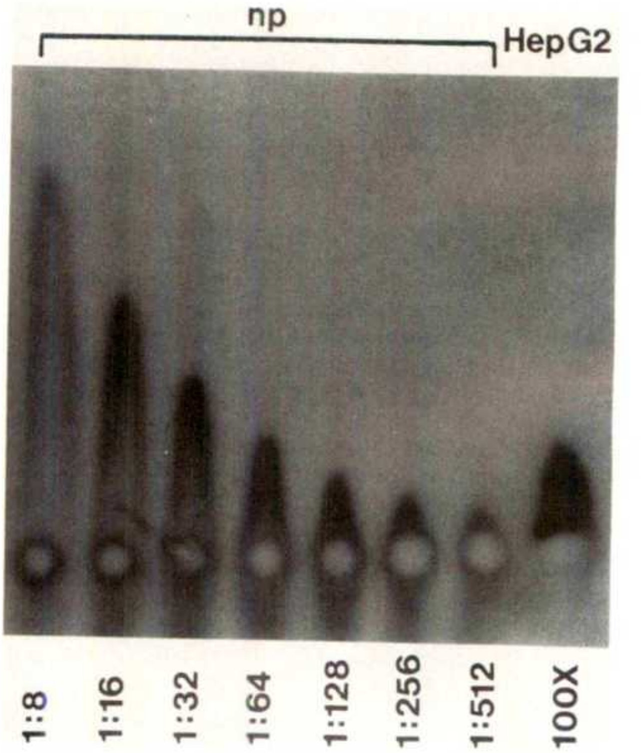

Fig 6. Quantification of FXIII in Hep G2 conditioned media by rocket immunoelectrophoresis. Rocket immunoelectrophoresis of dilutions of normal human plasma (np) and 100-fold-concentrated Hep G2 SFCM (10 µL/well). The gel contained anti-FXIII B antiserum at a concentration of 0.025%. The rockets were visualized with ^{125}I–protein A followed by autoradiography.

means of competitive radioimmunoassay based on specific radioimmunoassays for the A and B proteins of FXIII that were developed previously.[32,33] By radioimmunoassay (data not shown), the total concentration of FXIII antigen was found to be approximately 4 to 8 ng/mL in Hep G2 SFCM (unconcentrated), in good agreement with the results of the rocket immunoelectrophoresis procedure.

Altogether, these results clearly demonstrate synthesis and secretion of the FXIII B protein by both the Hep G2 and PLC/PRF/5 human hepatoma cell lines and suggest that these cells also synthesize the FXIII A protein and secrete a portion of the B protein as the plasma FXIII tetrameric complex A_2B_2.

DISCUSSION

The major focus of this work has been the demonstration and characterization of the FXIII synthesized and secreted by the cell line Hep G2. Hep G2 is a human hepatocellular carcinoma cell line that has morphologic characteristics similar to liver parenchymal cells and that appears to have normal biosynthetic and secretory capability.[20] It has been shown to synthesize a variety of coagulation and fibrinolytic proteins, including fibrinogen,[23] α_2-antiplasmin,[21] plasminogen activator,[22] prothrombin, factor X, antithrombin III, factor V, and factor VII.[25-27] The Hep G2 proteins that have been examined in detail have the same structural and functional characteristics as their native plasma counterparts. In addition, Hep G2 cells have been shown to respond to vitamin K and to warfarin in regulating the synthesis and secretion of prothrombin[26] and to steroids in regulating fibrinogen synthesis.[24] It is therefore clear that the Hep G2 cell line has the capacity for normal synthesis of a number of coagulation proteins and the ability to respond appropriately to modulation by known pharmacological agents. This appears to be the most carefully characterized, biosynthetically normal hepatocyte cell line available. The very low plasma concentration and long half-life of FXIII made it important to begin studies with a cell line possessing these characteristics. PLC/PRF/5 is another unrelated, human hepatocellular carcinoma cell line that, in contrast to Hep G2, shows a very different pattern of secreted proteins.[20] PLC/PRF/5 does not secrete fibrinogen or albumin. From the studies described here, it is clear that both of these human hepatoma cell lines synthesize and secrete the FXIII B protein. Previously, methods similar to those used in this study were employed in analysis of the human monocyte-macrophage cell line U-937[12] and human monocytes and macrophages.[14] No B-chain material could be detected in these cell extracts, whereas A-chain material was readily visualized.[12,14]

Previously, some studies have suggested that FXIII is synthesized by hepatocytes. FXIII has been reported to be in the cytoplasm of liver parenchymal cells by both fluorescent and immunoperoxidase techniques.[16,17] However, characterization of the antibodies used in these studies was not reported. In one case, all parenchmal cells appeared to be positive, raising a question of antibody specificity. Given the concentration and half-life of FXIII in plasma, one would expect cellular detection to be below the sensitivity of fluorescence microscopy. With specific antisera to A and B proteins, neither we nor others[45] have been able to demonstrate convincing, reproducible staining by immunofluorescence in hepatocytes. In addition, the Hep G2 cells appear negative for FXIII by immunofluorescence studies (data not shown). This argues for the use of more sensitive biochemical methods of approach rather than the reliance on a single method of immunohistochemistry with its problems of antibody specificity and sensitivity.

To date there has been no direct demonstration of FXIII synthesis and secretion by hepatocytes. Previous evidence based on clinical data documents reduced levels of plasma FXIII concomitant with liver disease in some patients but not in others,[1] but in fact, a decrease in plasma FXIII correlates with the severity of the systemic illness rather than with any specific illness.

In this work, FXIII B protein has been shown to be synthesized and secreted by a well-characterized hepatoma cell line that is known to secrete many of the proteins produced by normal, nonmalignant hepatocytes and also by a less well-characterized hepatoma cell line with a different pattern of secreted proteins. In our study, FXIII B protein has been immunochemically identified in the SFCM of these cell lines. This FXIII B protein was found to be antigenically identical to that of the human plasma FXIII B chain. This study represents the first detection of the B protein in any cell line by biosynthetic methods and the first biochemical demonstration of a site of synthesis of the plasma FXIII B chain.

The results of the CIE studies using normal plasma and purified FXIII A_2B_2, are interpreted as suggesting that the Hep G2 cell line also synthesizes and secretes at least a portion of the B protein as part of the A_2B_2 complex. Radiolabeling of a precipitin arc generated by CIE is not, in itself, conclusive evidence of the synthesis of a specific protein, since binding of secreted proteins to antigen-antibody precipitates or to proteins present in the normal plasma used as a carrier could give false-positive results. Experiments have shown[33] that when either A or B protein is present in excess it is loosely bound to the zymogen complex Results of our independent agarose gel electrophoresis experiments support our conclusions concerning the presence of both A and B proteins in the SFCM; however, confirmation of the prescence of the FXIII A_2B_2 complex awaits results of additional metabolic labeling experiments.

Despite repeated efforts to determine the mol wt of the A protein synthesized by Hep G2, visualization by immunoblotting of any specific A protein band in SDS-PAGE at the appropriate mol wt was unsuccessful. Further investigation indicated that the sensitive A protein had probably undergone proteolytic cleavage in the Hep G2 SFCM. Since the specific anti-FXIII A antibodies have a decreased capacity to bind to degraded FXIII A protein (as evidenced by a lack of precipitability of activated FXIII A_2 by these antibodies in both rocket immunoelectrophoresis and CIE [unpublished observations]), detection under these circumstances would be difficult. The results using nondenaturing agarose gel electrophoresis suggest A protein degradation, and possible B protein degradation, in Hep G2 SFCM upon storage. The mobility of the degraded A protein in the agarose gel is similar to that of the thrombin-activated A protein.[44] Evidence for the presence of proteolytic activity in Hep G2 culture medium exists from the work of others.[26] Factor X was isolated from Hep G2 conditioned medium in both a single-chain form and a two-chain form. The postsecretion cleavage of factor X into the two-chain form was thought to be due to the presence of a serine protease in the Hep G2 conditioned medium that could be inhibited by the addition of 1 mmol/L benzamidine to the conditioned medium. Addition of several inhibitors to our cultures (such as 1 mmol/L EDTA) did not permit the detection of FXIII A protein in the SFCM by immunoblotting following SDS-PAGE but did facilitate detection of the A protein from the cell lysate in the agarose gels. FXIII is also known to be labile to degradation by granulocyte proteases such as cathepsin C.[46,47] The ability to detect A protein in the SFCM (even in its degraded form) by the technique of agarose gel electrophoresis is probably due to the fact that this type of electrophoresis is performed under nondenaturing conditions. The method as described represents a form of immunochemical analysis that does not rely on immunofixation within the agarose gel or on immunoprecipitation prior to electrophoretic analysis. The absence of any SDS permits the antigen to be detected in its nondenatured state. It is clear that the anti-FXIII A antibodies are conformationally dependent.

Conclusions derived from our in vitro data may not necessarily reflect the complete in vivo scenario. Extrapolation of the synthesis of FXIII to normal human parenchymal cells on the basis of the ability of the Hep G2 cells and PLC/PRF/5 cells to secrete this protein should be made with some caution, since both cell lines originate from transformed cells. The results of the nondenaturing agarose gel electrophoresis experiments suggest that between the time of synthesis and secretion the FXIII A protein may undergo processing by Hep G2 cells, which is not equivalent to processing by PLC/PRF/5 cells. However, the evidence presented here suggests that the human hepatocyte possesses the ability to produce plasma FXIII in vivo. Recently, the primary structure of human FXIII B subunit was determined using a cDNA library prepared from human liver mRNA.[48] Assuming the rate of secretion of FXIII by these transformed cells (0.15 ng/10^6 cells/hour) is similar to the rate of secretion by normal hepatocytes, liver synthesis is sufficient to account for the amount of FXIII present in plasma This calculation is based on a similar calculation for factor V[25] and assumes that the plasma volume is 2.8 L, plasma FXIII concentration is 30 μg/mL,[31] the $t_{1/2}$ of FXIII is 8.4 days,[17] and the adult liver contains 3×10^{11} hepatocytes.[49] The rate of secretion of FXIII was estimated from our results for the amount of FXIII that accumulated over a three-day period from a confluent culture of the Hep G2 cells

The control mechanisms for secretion in vivo remain to be definitely demonstrated, but present clinical evidence reflects the control of FXIII B protein expression by the levels of circulating FXIII A protein, at least in the cases of FXIII-deficient patients.[50] In addition, patients who are congenitally deficient in FXIII have little or no detectable A protein and about 50% B protein, whereas heterozygotes have about 50% A and 80% B protein. The role of this free B protein has not been clearly defined. Current experiments are in progress to use the Hep G2 cells to address questions of metabolism as well as to elucidate the steps involved in the processing, assembly, and secretion of the FXIII A_2B_2 complex.

REFERENCES

1 Lorand L, Losowsky MS, Miloszewski KJM: Human factor XIII. Fibrin-stabilizing factor. Prog Hemost Thromb 5:245, 1980

2. McDonagh J: Structure and function of factor XIII, in Coleman RW, Hirsh J, Marder VJ, Salzman EW (eds)· Hemostasis and Thrombosis. Basic Principles and Clinical Practice. Philadelphia, Lippincott, 1982, p 164

3. Grinnell F· Fibronectin and wound healing. J Cell Biochem 26:107, 1984

4 Dvilansky A, Britten AFH, Loewy AG· Factor XIII assay by an isotope method I Factor XIII (transamidase) in plasma, serum, leukocytes, erythrocytes and platelets and evaluation of screening tests of clot solubility. Br J Haematol 18:399, 1970

5. Schwartz ML, Pizzo SV, Hill RL, McKee PA. Human Factor XIII from plasma and platelets: Molecular weights, subunit structure, proteolytic activation and crosslinking of fibrinogen and fibrin. J Biol Chem 248 1395, 1973

6 Chung SI, Lewis MS, Folk JE. Relationships of the catalytic properties of human plasma and platelet transglutaminases (activated blood coagulation factor XIII) to their subunit structure. J Biol Chem 249·940, 1974

7. Curtis CG, Brown KL, Credo RB, Domanik RA, Gray A, Stenberg P, Lorand L: Calcium dependent unmasking of active center cysteine during activation of fibrin stabilizing factor Biochemistry 13·3774, 1974

8 Freyssinet JM, Lewis BA, Holbrook JJ, Shore JD: Protein-protein interactions in blood clotting. The use of polarization of fluorescence to measure the dissociation of plasma factor XIIIa. Biochem J 169·403, 1978

9 Kiesselback TH, Wagner RH: Demonstration of factor XIII in human megakaryocytes by a fluorescent antibody technique. Ann NY Acad Sci 202:318, 1972

10. Chung SI. Multiple molecular forms of transglutaminases in human and guinea pig, in Markert CL (ed): Isozymes, vol 1 Orlando, FL, Academic, 1975, p 259

11. Bohn H, Haupt H, Kranz T· Die molekulare Struktur der fibrinstabilisierenden Faktoren des Menschen. Blut 25:235, 1972

12. Colvin R, Lynch G, McDonagh J, Erikson M, Karnick J, Power K, Kradin R: Factor XIII A subunit is synthesized and expressed by macrophages. Fed Proc 44:906, 1985

13. Muszbek L, Adany R, Szeyedi G, Polgar J, Kavai M· Factor XIII of blood coagulation in human monocytes Thromb Res 37:401, 1985

14. Henriksson P, Becker S, Lynch G, McDonagh J· Identification of Intracellular factor XIII in human monocytes and macrophages. J Clin Invest 76.528, 1985

15 Workman EF Jr, Lundblad RL: The role of the liver in the biosynthesis of the non–vitamin K–dependent clotting factors Semin Thromb Hemost 4 15, 1977

16 Lee SY, Chung SI. Biosynthesis and degradation of plasma protransglutaminase (factor XIII). Fed Proc 35·1486, 1976

17 Ikematsu S: An approach to the metabolism of factor XIII. Acta Haematol Jpn 44:1499, 1981

18. Fear JD, Jackson P, Gray C, Miloszewski KJA, Losowsky MS: Localization of factor XIII in human tissues using an immunoperoxidase technique. J Clin Pathol 37:560, 1984

19. Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB: Controlled synthesis of HBsAg in a differentiated human liver carcinoma–derived cell line. Nature 282:615, 1979

20. Knowles BB, Howe CC, Aden DP: Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 209 497, 1980

21. Fair DS, Plow EF: Synthesis and secretion of the fibrinolytic components, including α_2-antiplasmin, by a human hepatoma cell line. J Lab Clin Med 101.372, 1983

22. Levin EG, Fair DS, Loskutoff DJ: Human hepatoma cell line plasminogen activator. J Lab Clin Med 102:500, 1983

23 Yu S, Sher B, Kudryk B, Redman CM: Intracellular assembly of human fibrinogen. J Biol Chem 258 13407, 1983

24. Yu S, Sher B, Kudryk B, Redman CM: Fibrinogen precursors. Order of assembly of fibrinogen chains. J Biol Chem 259:10574, 1984

25 Wilson DB, Salem HH, Mruk JS, Maruyama I, Majerus PW: Biosynthesis of coagulation factor V by a human hepatocellular carcinoma cell line J Clin Invest 73:654, 1984

26. Fair DS, Bahnak BR. Human hepatoma cells secrete single chain factor X, prothrombin and antithrombin III. Blood 64.194, 1984

27. Fair DS, Marlar RA. Biosynthesis and secretion of factor VII, protein C, protein S, and the protein C inhibitor from a human hepatoma cell line. Blood 67:64, 1986

28 Morris KM, Aden DP, Knowles BB, Colten HR: Complement biosynthesis by the human hepatoma-derived cell line Hep G2 J Clin Invest 70:906, 1982

29. Goldberger G, Arnaout MA, Aden D, Kay R, Rits M, Colten HR· Biosynthesis and postsynthetic processing of human C3b/C4b inactivator (factor I) in three hepatoma cell lines J Biol Chem 259·6492, 1984

30 Kazama M, McDonagh J, Wagner RH, Langdell RD, McDonagh RP. Purification and immunochemical characterization of human plasma factor XIII Haemostasis 5:329, 1976

31. McDonagh J, Waggoner WG, Hamilton EG, Hindenach B, McDonagh RP. Affinity chromatography of human plasma and platelet factor XIII on organomercurial agarose Biochim Biophys Acta 446:345, 1976

32. Skrzynia C, Reisner HM, McDonagh J: Characterization of the catalytic subunit of factor XIII by radioimmunoassay. Blood 60·1089, 1982

33 Ikematsu S, McDonagh RP, Reisner HM, Skrzynia C, McDonagh J: Immunochemical studies of human factor XIII. Radioimmunoassay for the carrier subunit of the zymogen. J Lab Clin Med 97:662, 1981

34. Hubbard AL, Cohn ZA· The enzymatic iodination of the red cell membrane. J Cell Biol 55:390, 1972

35 Alexander JJ, Bey EM, Geddes EW, Lecatsas G: Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 50:2124, 1974

36. Laemmli UK Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227.680, 1970

37. Towbin H, Staehlin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets Procedure and some applications. Proc Natl Acad Sci USA 76.4350, 1979

38. Johnson DA, Elder JH. Antibody directed to determinants of a Moloney virus–derived MCF GP70 recognizes a thymic differentiation antigen J Exp Med 159.1751, 1983

39. Ganrot PO: Crossed immunoelectrophoresis Scand J Clin Lab Invest 29:39, 1972 (suppl 124)

40. Grubb AO Crossed immunoelectrophoresis. Scand J Immunol 17:113, 1983 (suppl 10)

41. Johansson BG: Agarose gel electrophoresis. Scand J Clin Lab Invest 29:7, 1972 (suppl 124)

42. McMichael JC, Greisiger LM, Millman I: The use of nitrocellulose blotting for the study of hepatitis B surface antigen electrophoresed in agarose gels J Immunol Methods 45:79, 1981

43. Laurell C: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 15:45, 1966

44 Henriksson P, McDonagh J: Factor XIII activation and interactions, in Egbring R, Klingemann HG (eds): Factor XIII and Fibronectin Marburg, Germany, Med Verlagsgesellschaft, 1983, p 1

45. Adany R, Kiss A, Thomazy V, Muszbek L: Origin of human factor XIII Thromb Haemost 54.147, 1985

46 Henriksson P, Nilsson IM, Ohlsson K, Stenberg P Granulocyte elastase activation and degradation of factor XIII. Thromb Res 18:343, 1980

47. Klingemann HG, Egbring R, Holst F, Gramse M, Havemann K: Degradation of human plasma fibrin stabilizing factor XIII subunits by human granulocytic proteinases. Thromb Res 28:793, 1982

48. Ichinose A, McMullen B, Fujikawa K, Davie EW: Primary structure of human factor XIII B subunit: A protein with ten tandem repeats. Fed Proc 45:1636, 1986

49 Gates GA, Henley KS, Pollard HM, Schmidt E, Schmidt FW: The cell populations of human liver J Lab Clin Med 57:182, 1961

50. Rodeghiero F, Morbin M, Barberi T· Subunit A of factor XIII regulates subunit B plasma concentration. Thromb Haemost 46:621, 1981

Proteolysis of Platelet Glycoprotein Ib by Plasmin Is Facilitated by Plasmin Lysine-Binding Regions

By Burt Adelman, Alan D. Michelson, Joshua Greenberg, and Robert I. Handin

We have characterized the effects of plasmin on glycoprotein Ib (GpIb), a platelet membrane receptor for von Willebrand factor (vWF), and on glycocalicin, a fragment of the α chain of GpIb that contains the vWF-binding region. The addition of 4.5×10^{-7} mol/L plasmin to washed platelets caused a time-dependent decrease in ristocetin-induced, vWF-dependent platelet agglutination. ϵ-Aminocaproic acid (EACA) inhibited plasmin release of glycocalicin-related antigen from washed platelets and preserved vWF-dependent platelet agglutination, thus indicating that the lysine-binding sites on plasmin facilitated its degradation of GpIb. To demonstrate a direct interaction between plasmin and the vWF-binding region of GpIb we incubated purified glycocalicin with plasmin. Plasmin degraded the glycocalicin into two small carbohydrate-poor peptides and into a larger carbohydrate-rich fragment. EACA was able to inhibit plasmin-mediated degradation of glycocalicin in a concentration-dependent fashion. These studies indicated that plasmin degradation of GpIb was due to a direct interaction between plasmin and GpIb and that this effect was mediated by the lysine-binding region of the plasmin molecule.

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THE PLATELET surface protein glycoprotein Ib (GpIb) has a molecular weight (mol wt) of 170,000 and is composed of two disulfide-linked subunits, an α chain with a mol wt of about 145,000 and β chain with a mol wt of about 25,000.[1,2] Current evidence indicates that the α subunit contains an important platelet-binding site for von Willebrand factor (vWF). Monoclonal antibodies and heteroantibodies directed against the GpIb α subunit block binding of vWF to platelets and prevent vWF-dependent, ristocetin-induced platelet agglutination.[2–4] The α chain may also provide a binding site that facilitates platelet activation by thrombin [4–6]

In previous studies we have demonstrated that plasmin can degrade GpIb and thereby interfere with platelet-vWF interactions.[7] This effect of plasmin on platelet agglutination corresponds to the plasmin-mediated release of a glycocalicin-related antigen from platelet GpIb. Glycocalicin is a heavily glycosylated fragment of the α chain of GpIb that contains the GpIb vWF-binding site [2–4,6] These in vitro findings are consistent with the clinical observations of Ordinas et al[8] and Castillo et al[9] that indicate that vWF-dependent platelet interactions are abnormal during pathological states of accelerated fibrinolysis.

Miles and Plow have recently demonstrated that specific binding of plasmin and plasminogen to platelets is mediated by lysine-binding sites associated with the kringle structures on the heavy-chain region of plasmin(ogen).[10] In addition, their data indicate that thrombin activation of platelets enhances binding and that lysine analogues inhibit it. Although little is known about the effects of the fibrinolytic system on platelet function, such interactions may play a role in promoting the anticoagulant state and/or hemorrhagic complications associated with systemic fibrinolysis. The purpose of our study was to further evaluate the action of plasmin on GpIb and to better define the nature of this platelet-plasmin interaction.

MATERIALS AND METHODS

Materials Sodium ^{125}I was purchased from New England Nuclear, Boston; ristocetin from Bio-Data Corp, Horsham, PA; and plasmin from KABI, Uppsala, Sweden through Helena Laboratories, Beaumont, TX. The plasmin was dissolved in 10 mmol/L Tris–0.15 mol/L NaCl, pH 7.4, and stored in aliquots at −40 °C. The final concentration of the stock solution was determined using the chromogenic substrate S-2288 (KABI through Helena Laboratories) [7] Aprotinin and ϵ-aminocaproic acid (EACA) were purchased from Sigma Chemical Co, St Louis Streptokinase was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. All chemicals were reagent grade

Washed human platelets. Venous blood was drawn into 3.8% sodium citrate (wt/vol). Nine-parts blood were added to one-part anticoagulant. After mixing, the blood was centrifuged at 200 g for ten minutes at room temperature and the upper two thirds of the platelet-rich plasma (PRP) collected An equal volume of washing buffer (10 mmol/L Tris, 0.15 mol/L NaCl, 20 mmol/L EDTA, pH 7.4) was added to the PRP and this mixture centrifuged at 3,200 g for 15 minutes at room temperature. The platelet pellet was resuspended in 10 mL of washing buffer and washed an additional three times. The platelets were finally suspended in the same buffer at a concentration of $1 \times 10^6/\mu$L. Platelets were counted in a Coulter Counter model F (Coulter Electronics, Hialeah, FL).

Effect of plasmin on ristocetin-induced, vWF-dependent platelet agglutination. Washed platelets, $1 \times 10^6/\mu$L in washing buffer, were incubated with plasma at room temperature At various time intervals, 200-μL aliquots of the incubation mixture were removed and aprotinin added to stop the reaction. The amount of aprotinin added was always in excess of the amount needed to totally inhibit

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plasmin activity in the S-2288–based assay.[7] The platelets were then centrifuged at 8,000 g in a Beckman Microfuge B (Beckman Instruments, Inc, Palo Alto, CA). The platelet pellets were resuspended in 1.5 mL of fresh platelet-poor plasma (PPP) prepared from the original donor. Samples of reconstituted PRP (400 μL) were placed in aggregometer cuvettes to study platelet agglutination, which was initiated by adding 50 μL of a 10 mg/mL solution of ristocetin to each PRP sample while it was stirred at 37 °C in a Payton Dual Channel Aggregometer (Payton Associates, Buffalo, NY).

Effect of EACA on plasmin digestion of platelet GpIb. Washed platelets, $1 \times 10^6/\mu$L, were suspended in washing buffer containing varying amounts of EACA. Each sample was incubated for 60 minutes at room temperature with 4.5×10^{-7} mol/L plasmin. Control samples contained no plasmin. At the end of the incubation period, excess aprotinin was added to each sample and the platelets and supernatants separated as described. The supernatants were assayed for glycocalicin-related antigen by enzyme-linked immunosorbent assay (ELISA) as previously described,[7] and the platelets were resuspended in PRP and assayed for ristocetin-induced agglutination. The ELISA assay utilizes the monoclonal antiglycocalicin antibody 6D1, which was generously provided by Dr Barry Coller, State University of New York, Stony Brook.

Plasmin digestion of purified platelet glycocalicin. Platelet glycocalicin was purified as previously described.[7] Glycocalicin, 8.2×10^{-6} mol/L, in TBS (0.01 mol/L Tris, 0.015 mol/L NaCl, pH 7.4) was incubated with 7×10^{-7} mol/L plasmin. At various time periods aliquots were removed from the incubation mixture, combined with an equal volume of nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and boiled for three minutes. Control samples contained no plasmin. Each sample was subjected to SDS-PAGE on 7.5% gels.[11] For each experiment two identical gels were produced so that one could be stained with Coomassie blue and the other with periodic acid–Schiff (PAS) reagent.

To further study the effect of EACA on plasmin digestion of glycocalicin we used ^{125}I-labeled glycocalicin. Glycocalicin was iodinated using Iodobeads following the manufacturer's suggested technique (Pierce Chemical Co, Rockford IL). Plasmin, in TBS, was incubated with ^{125}I-glycocalicin and varying amounts of EACA. The incubations were stopped after 60 minutes by boiling in SDS-PAGE sample buffer. Aliquots of each specimen were subjected to SDS-PAGE on 10% gels followed by autoradiography. To assure comparability, samples applied to the gels contained identical counts per minute. Autoradiograms of the dried gels, made by exposing them to x-ray film (Kodak, Rochester, NY) at −20 °C, were analyzed by an integrating scanning densitometer (Beckman model CDS-200). Results were expressed as the percentage of the total ^{125}I-glycocalicin remaining after digestion.

RESULTS

Plasmin digestion of platelet GpIb and inhibition of vWF-dependent platelet agglutination. Figure 1 shows the response to ristocetin of washed platelets that had been incubated with 4.5×10^{-7} mol/L plasmin and then added back to PPP. Plasmin treatment of these platelets caused a progressive decline in the rate of agglutination so that by 90 minutes agglutination was totally inhibited.

To determine whether plasmin interacts directly with GpIb and whether this interaction is near the vWF-binding region we examined its effect on purified glycocalicin. As seen in Fig 2, plasmin degraded glycocalicin into three major fragments. The two low mol wt peptides (less than 45,000 mol wt) contained relatively little carbohydrate as determined by PAS staining following SDS-PAGE. The third, the highest mol wt fragment, was heavily glycosylated and could only be visualized on the PAS-stained gels.

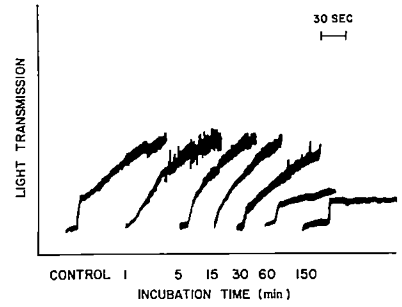

Fig 1. Effect of plasmin on ristocetin-induced, vWF-dependent platelet agglutination. Washed platelets, $1 \times 10^6/\mu$L, were incubated with 4.5×10^{-7} mol/L plasmin. At various time points samples were removed, excess aprotinin added, and the platelets separated by centrifugation. The sedimented platelets were resuspended in PPP obtained from the same donor, after which, ristocetin, 1.25 mg/mL, was added to induce agglutination. The control sample was not incubated with plasmin.

Recently it has been demonstrated that plasminogen can bind to thrombin-stimulated platelets and be activated by tissue plasminogen activator.[10,12] To confirm that release of glycocalicin-related antigen in our experiments was due only to the addition of exogenous plasmin, we measured platelet agglutination after treating washed platelets with streptokinase for one hour. Neither 10,000 IU/mL nor 50,000 IU/mL streptokinase affected vWF-dependent, ristocetin-induced platelet agglutination (data not shown). We therefore concluded that plasminogen binding to platelets did not occur during the blood-drawing or platelet-washing procedures used in our experiments.

Effect of EACA on plasmin digestion of GpIb and glycocalicin. We next studied the effect of EACA on plasmin inhibition of ristocetin-induced platelet agglutination. As can be seen in Fig 3, the addition of EACA in concentrations greater than 10^{-4} mol/L preserved ristocetin-induced agglutination of plasmin-treated platelets. To directly examine the inhibitory effect of EACA on plasmin digestion of GpIb we measured the release of glycocalicin-related antigen from plasmin-treated platelets. The results of these studies, summarized in Fig 4, indicated that EACA inhibited plasmin degradation of GpIb in a dose-dependent manner, with 50% inhibition occurring at an approximate concentration of 5×10^{-4} mol/L EACA.

We also examined the effect of EACA on plasmin digestion of purified, ^{125}I-labeled glycocalicin. ^{125}I-glycocalicin was incubated with 7×10^{-7} mol/L plasmin and various concentrations of EACA. After incubation for 60 minutes, aliquots of the reaction mixtures were subjected to SDS-PAGE and autoradiography. The autoradiograms were analyzed by scanning densitometry and results expressed as the percentage of the total ^{125}I-glycocalicin added to the incubation mixture still present after plasmin treatment. As can be seen in Table 1, EACA inhibited plasmin digestion of ^{125}I-glycocalicin in a dose-dependent fashion.

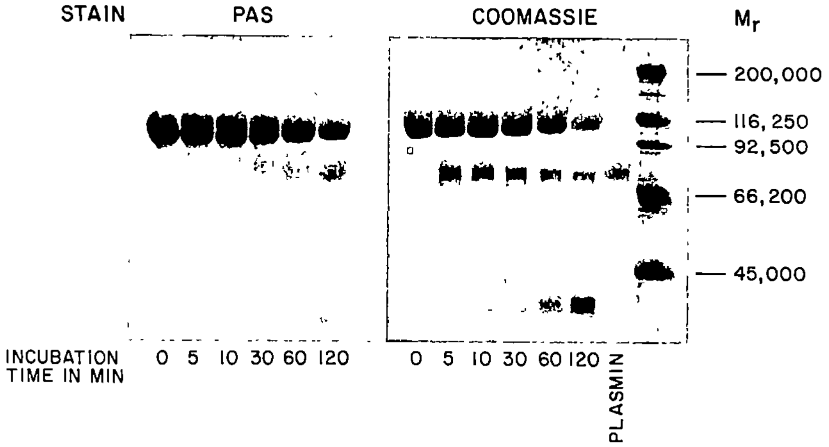

Fig 2. Effect of plasmin on glycocalicin. Purified glycocalicin was incubated with plasmin for various time periods, after which the reaction was stopped by boiling in SDS-PAGE buffer (see Materials and Methods) Each sample was then subjected to SDS-PAGE on 7.5% gels. Parallel gels were run so that each sample could be stained with Coomassie blue and PAS reagent. The lane marked "plasmin" contains plasmin only and is present to indicate its location in the Coomassie blue–stained gel.

DISCUSSION

In this study we report that plasmin proteolysis of platelet GpIb occurs in the vicinity of its vWF-binding site. In addition, we observed that this effect could be inhibited in a dose-dependent fashion by the lysine analogue EACA, thus indicating that the lysine-binding regions of plasmin facilitate its degradation of GpIb. Digestion of GpIb is accompanied by release of glycocalicin-related antigen from the platelet surface and by diminished vWF-dependent platelet agglutination.

The lysine-binding regions present on the kringle rings of plasmin coordinate its binding to fibrin and fibrinogen and therefore mediate its fibrinolytic action.[12,13] Lysine analogues such as EACA and tranexamic acid bind to these regions and inhibit fibrinolysis by preventing contact between plasmin and fibrin(ogen).[14] Miles and Plow have demonstrated that plasminogen and plasmin binding to platelets is also dependent upon these lysine-binding regions and can be inhibited by lysine analogues.[10,15] Our studies indicate that GpIb is a plasmin interaction site on the platelet membrane and that plasmin-GpIb interactions are analogous to fibrin(ogen)–plasmin interactions.

Because fibrin and fibrinogen can be present on the platelet surface and are known to bind plasmin and plasminogen, we conducted our experiments under conditions that would minimize platelet-bound fibrinogen.[16-19] We utilized resting platelets suspended in a fibrinogen-free buffer that contained an adequate amount of EDTA to prevent attachment of secreted fibrinogen. Thus, plasmin degradation of GpIb appears to result from a direct interaction between these two proteins on the platelet surface. These observations are consistent with those of Miles and Plow, who have demonstrated that plasminogen binding to resting platelets is not facilitated by fibrinogen.[10]

Platelets contain a calcium-dependent protease that can degrade GpIb and release glycocalicin.[20,21] To demonstrate that the effect of plasmin on platelet-bound GpIb was direct and not a result of plasmin-induced release of calcium protease, we examined the effect of plasmin on purified glycocalicin. We found that plasmin was able to induce proteolysis of glycocalicin rapidly into three major fragments. One fragment was carbohydrate rich and the others carbohydrate poor. We suspect that the smaller of the carbohydrate-poor fragments was derived from the larger one because the combined mol wt of all three fragments exceeds that of glycocalicin. However, because of the aberrant behavior of glycosylated proteins in SDS-PAGE[22] we cannot estimate with certainty the mol wt of the large carbohydrate-rich fragment, and it remains possible that all

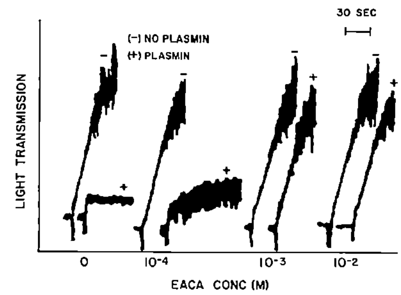

Fig 3. Effect of EACA on plasmin-mediated reduction of ristocetin-induced platelet agglutination. Platelets, 1 × $10^6/\mu L$, were incubated with 4.5 × 10^{-7} mol/L plasmin and varying amounts of EACA for 60 minutes. At the end of the incubation aprotinin was added to the platelet suspension and the platelets separated by centrifugation. The platelet pellets were resuspended in PPP and ristocetin, 1.25 mg/mL, was added to induce agglutination. The corresponding control platelets were incubated with EACA but not with plasmin.

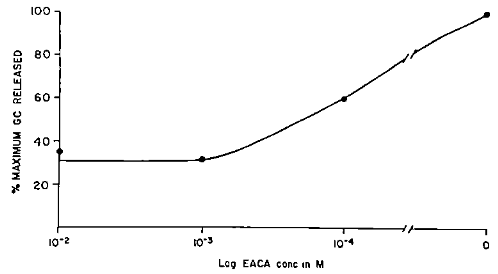

Fig 4. Effect of EACA on plasmin-dependent release of glycocalicin-related antigen from washed platelets. Platelets, 1 × $10^6/\mu L$, were suspended in buffer containing 4.5 × 10^{-7} mol/L plasmin and varying concentrations of EACA. After a 60-minute incubation period, excess aprotinin was added to each sample and the platelets pelleted by centrifugation. The glycocalicin content (GC) of the supernatants was determined by ELISA. Each point represents the mean of two determinations.

Table 1. Effect of EACA on Plasmin Digestion of ^{125}I-Glycocalicin

EACA Concentration (mol/L)	Residual ^{125}I-Glycocalicin (%)
0	52
1×10^{-5}	54
1×10^{-4}	61
7.5×10^{-4}	64
1×10^{-3}	70
1×10^{-2}	81
No plasmin, no EACA	100

three pieces were derived directly from glycocalicin. These experiments further demonstrate that plasmin can act specifically on GpIb and confirm that it is able to degrade the GpIb-vWF–binding region.

Other investigators have reported significant effects of plasmin on platelet function and platelet membrane glycoprotein content. Winocour et al observed shortened platelet survival in rats with a catheter-induced arterial injury and demonstrated that treatment of these animals with EACA normalized platelet survival and promoted increased thrombus formation.[23] These observations suggested to the authors that plasmin, generated in response to vascular injury, modified circulating platelets in a way that prevented them from contributing to thrombus formation and promoted their rapid clearance. Data from Greenberg et al support this argument. They demonstrated that plasmin treatment of rabbit platelets in vitro reduced membrane glycoprotein content and resulted in shortened survival upon their reinfusion into normal animals [24]

Plasmin can also induce changes in platelet metabolic function. Niewiarowski et al[25] demonstrated that plasmin treatment of platelets induced the release of stored adenine nucleotides and diminished their aggregation response to adenosine diphosphate. Shafer and Adelman observed that plasmin treatment of gel-filtered platelets caused inhibition of platelet aggregation in response to thrombin, calcium ionophore, and collagen in parallel with the inhibition of platelet thromboxane production.[26] The authors suggested that plasmin treatment of platelets blocked mobilization of endogenous arachidonic acid from platelet membrane pools.

The results of this study and those mentioned above indicate that platelet–plasmin interactions may have significant effects on platelet function. Plasmin-induced platelet inhibition occurring in association with fibrin(ogen) degradation may be an important action of endogenous fibrinolysis or fibrinolytic therapy. We feel that these observations suggest the need for further in vivo study of platelet function during systemic fibrinolysis.

REFERENCES

1 Phillips DR· An evaluation of membrane glycoproteins in platelet adhesion and aggregation, in Spaet T (ed) Progress in Hemostasis and Thrombosis, vol 5. Orlando, FL, Grune & Stratton, 1980, p 81

2. Clemetson KJ, Naim HY, Luscher EF· Relationship between glycocalicin and glycoprotein Ib of human platelets. Proc Natl Acad Sci USA 78:2712, 1981

3 Coller BS, Peerschke EI, Scudder LE, Sullivan CA: Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets. Additional evidence in support of gpIb as a platelet receptor for von Willebrand factor. Blood 61:99, 1983

4. Jenkins CSP, Clemetson KJ, Ali-Briggs EF: Antibodies against platelet membrane glycoproteins: III. Effect on thrombin and bovine von Willebrand factor-induced aggregation. Br J Haematol 53.491, 1983

5 Cooper HA, Bennet WP, White III GC, Wagner RH· Hydrolysis of human platelet membrane glycoproteins with a *Serratia marcescens* metalloprotease: Effect on response to thrombin and von Willebrand factor. Proc Natl Acad Sci USA 79:1433, 1982

6. Okumura T, Jamieson GA: Platelet glycocalicin, a single receptor for platelet aggregation induced by thrombin or ristocetin. Thromb Res 8:701, 1976

7. Adelman B, Michelson AD, Loscalzo J, Greenberg J, Handin RI: Plasmin effect on platelet glycoprotein Ib–von Willebrand factor interactions. Blood 65.32, 1985

8 Ordinas A, Margall S, Castillo R, Nurden AT: A glycoprotein I defect in the platelets of three patients with severe cirrhosis of the liver Thromb Res 13.297, 1978

9. Castillo R, Maragall S, Rodes J, Clemente C, Profitos J, Ordinas A Increased factor VIII complex and defective ristocetin-induced platelet aggregation in liver disease. Thromb Res 11:899, 1979

10. Miles LA, Plow EF: Binding and activation of plasminogen on the platelet surface J Biol Chem 260:4303, 1985

11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T Nature 227:680, 1970

12. Collen D: On the regulation and control of fibrinolysis. Thromb Haemost 43:77, 1980

13. Robbins RC: The plasminogen-plasmin enzyme system, in Coleman RW, Hirsh J, Marder VJ, Salzman EW (eds)· Hemostasis and Thrombosis. Philadelphia, Lippincott, 1982 p 623

14. Marder VJ, Butler FO, Barlow GH. Antifibrinolytic therapy, in Coleman RW, Hirsh J, Marder VJ, Salzman EW (eds): Hemostasis and Thrombosis. Philadelphia, Lippincott 1982, p 640

15. Miles LA, Plow EF: Platelet-plasminogen interaction: A potential role in fibrinolysis. Blood 62·305, 1983 (suppl 1)

16. Coller BS, Peerschke E, Scudder LE, Sullivan CA: A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic like state in normal platelets and binds to glycoprotein IIb and/or IIIa J Clin Invest 72:325, 1983

17. Nachman RL, Leung LLK: Complex formation of platelet membrane glycoproteins IIb-IIIa with fibrinogen J Clin Invest 69·263, 1982

18. Hawiger J, Kloczewiak M, Timmons S, Strong D, Doolittle RF Interaction of fibrinogen with staphylococcal clumping factor and with platelets, in Mosesson MW, Doolittle RF (eds): Molecular Biology of Fibrinogen and Fibrin. Ann NY Acad Sci 408:521, 1983

19 Marquerie GA, Plow EF, Edgington TS: Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 254·5357, 1979

20. Truglia JA, Straucher A. Purification and characterization of a calcium dependent sulfhydryl protease from human platelets. Biochem Biophys Res Commun 100:814, 1981

21. Phillips DR, Jakabova M: Ca^{2+}-dependent protease in human platelets. J Biol Chem 253:3435, 1978

22. Segrest JP, Jackson RL, Andrews EP, Marchesi VT: Human erythrocyte membrane glycoprotein· A re-evaluation of the molecu-

lar weight as determined by SDS polyacrylamide gel electrophoresis. Biochem Biophys Res Commun 44:390, 1971

23. Winocour PD, Kinlough-Rathbone RL, Richardson M, Mustard JF: Reversal of shortened platelet survival in rats by the antifibrinolytic agent, epsilon amino caproic acid. J Clin Invest 71 159, 1983

24. Greenberg JP, Packham MA, Guccione MA, Rand ML, Reimers HJ, Mustard JF: Survival of rabbit platelets treated in vitro with chymotrypsin, plasmin, trypsin, or neuraminidase Blood 53:916, 1979

25. Niewiarowski S, Senyi AF, Gillies P: Plasmin-induced platelet aggregation and platelet release reaction J Clin Invest 51 1647, 1973

26. Schafer A, Adelman B. Plasmin inhibition of platelet function and of arachidonic acid metabolism. J Clin Invest 75.446, 1985

Rh Blood Group–Specific Antibodies in Immune Hemolytic Anemia Induced by Nomifensine

By A. Salama and C. Mueller-Eckhardt

Nomifensine (Merital, Alival; Hoechst, Frankfurt, FRG), an antidepressant drug, may cause immune hemolytic anemia (IHA) of the so-called immune complex type that is believed to occur by means of an innocent-bystander mechanism. In this report we describe findings that are not consistent with this mechanism in a patient with nomifensine-induced intravascular IHA associated with renal failure. In vitro studies showed a transitory positive direct antiglobulin test (DAT) due to IgG, IgM, and C3 fixation. The causative antibodies were found to be (1) a drug-independent IgM antibody in the serum and eluate that reacted only with E-positive RBC, although the patient's RBC were E-negative; (2) an IgG antibody in the serum and initial eluates that showed a stronger reaction with e-positive than with e-negative or Rh_{null} RBC, but only in the presence of ex vivo antigen (ie, urine containing the drug and all its metabolities); and (3) an IgM antibody in the serum and initially also on the patient's RBC that, in the presence of ex vivo antigen as well as in the presence of known metabolites of the drug, agglutinated all RBC equally strongly, but was hemolytically more active against E-positive than E-negative cells. Within a few days of stopping the drug the hemolysis rapidly resolved without administration of prednisone, the DAT became negative with anti-IgG and anti-IgM, and the drug-independent anti-E disappeared, but both metabolite-dependent antibodies remained detectable in the patient's serum. We conclude that the production and specificity of the causative antibodies in this case were controlled by a larger antigenic site, presumably consisting of the drug and/or its metabolites plus RBC antigens, rather than by epitopes of the drug or metabolites alone.

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SEVERAL CASES of nomifensine-dependent immune hemolytic anemia (IHA) have been reported in the last 5 years.[1–6] It is generally maintained that the RBC in these patients are damaged by the so-called immune-complex mechanism. In this type of reaction, drugs are believed to serve as haptens by conjugating with some soluble noncellular macromolecule, giving rise to subsequent formation of high-affinity antibodies against the drug. The antibodies could first react with the drug, and then the antibody-drug complexes could be adsorbed nonspecifically by the RBC, which act merely as "innocent bystanders." This hypothesis is based on the assumption that immunization does not require conjugation of the drug with RBC and that the antibodies produced do not have specificity for red cell antigens.[7]

We have recently reported that nomifensine-dependent antibodies probably bind to target RBC via their Fab and not by their Fc portion by showing that, first, epitopes on the Fc portion of RBC-bound antibodies are still accessible to Fc-specific anti-IgG/IgM and, second, that nomifensine-dependent antibodies remain firmly attached to RBC if trace amounts of drug are added to the wash solution.[6] Such findings are not consistent with the aforementioned mechanism of nonspecific adsorption of drug-antibody complexes on target RBC.

We now report a patient with nomifensine-dependent IHA who, in the course of the disease, developed various drug-induced antibodies with Rh specificity: a transient IgM pseudo–anti-E (mimicking an alloantibody), a metabolite-dependent IgG anti-e (detectable in serum and eluates), and a metabolite-dependent IgM anti-E that was hemolytic. These findings provide direct evidence that the epitopes seen by the nomifensine-induced antibodies in this case are not formed solely by the drug or its metabolites but at least in part by RBC-specific membrane antigens.

CASE REPORT

K.I., a 58-year-old female, had been treated for psychotic episodes with various drugs since 1960. Nomifensine was first given to the patient in 1982. On November 5, 1985, nomifensine was readministered at a dose of 75 mg/d On November 19, 1985, she noticed weakness and jaundice. On the following day the patient was admitted to a local hospital for acute lumbar pain, malaise, and dark urine At initial presentation the patient showed the typical signs and symptoms of acute intravascular hemolysis: she was somnolent and extremely pale, and hemoglobinemia and hemoglobinuria were present. Her total hemoglobin concentration was 4 3 g/dL (free hemoglobin was not measured), the lactate dehydrogenase content was 1,746 (normal,<200 IU/L), the bilirubin level was 6.1 mg/dL (of which 4.3 mg was indirect bilirubin), and haptoglobin was undetectable Although the patient had never been transfused, the direct antiglobulin test (DAT) was found to be strongly positive, and an agglutinating anti-E was identified in the patient's serum. On the assumption of drug-dependent IHA, nomifensine was discontinued and the patient was transfused with 4 units of compatible, E-negative packed RBC Without further treatment, the hemolysis rapidly resolved, but transient renal insufficiency developed, requiring temporary hemodialysis. On December 4, 1985, the patient was transferred in good physical condition to a psychiatric hospital.

MATERIALS AND METHODS

Blood samples were collected from the patient on admission before any therapy and during hospitalization at times indicated in the Tables A complete serological analysis (ABO, Rh antigens, antibody screening) was made by conventional techniques For antibody identification the Resolve panel (Ortho Pharmaceutical Corp, Raritan, NJ) was used. Rh_{null} RBC were kindly provided by Professor S Seidl (Frankfurt). The DAT was assayed exclusively from EDTA-

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anticoagulated blood by a tube method using commercial antisera (polyspecific antiglobulin serum [Ortho], anti-IgG [Behringwerke AG, Marburg, FRG], anti-IgM and anti-IgA [Diamed, Morat, Switzerland], and anti-C3d [Dako, Copenhagen]). Antibodies were eluted from sensitized RBC by the heat technique (ten minutes at 56 °C) The detection of drug-dependent antibodies, including hemolysis assays, was performed as recently described[6] using nomifensine, its known three main metabolites (M1, M2, M3), and its ex vivo antigens (urine collected from a healthy volunteer before (U0, control) and five hours (U5) after the ingestion of 200 mg nomifensine)

To characterize the immunoglobulin classes of the antibodies, a serum sample of the patient obtained on day 15 was chromatographed on a Sephacryl S 300 column (Pharmacia Fine Chemicals, Uppsala, Sweden), at 4 °C. The immunoglobulin fractions (IgM, IgA, IgG) were concentrated to the original serum volume and examined for antibody activity against RBC in the presence and absence of the drug and its metabolites.

RESULTS

Demonstration of a transitory anti-E in early serum samples. The patient's blood group was O Rh-positive (CCDee). Her serum contained an agglutinating anti-e in initial samples (Table 1), but not in specimens taken after day 6. Although this antibody had a relatively low titer, its agglutinating activity against e-positive cells did not change after washing the cells three times with large volumes of saline so that the indirect antiglobulin test (IAT) could not be performed. This antibody was drug independent since its reactivity was unaffected when the serum was dialyzed in large volumes of saline containing 0.2% bovine serum albumin (saline-BSA) or when the antibody was recovered by elution after absorption on e-positive RBC in the absence of the drug and its metabolites.

Characterization of RBC-bound antibodies in the DAT. The DAT was performed with RBC obtained from the patient before any treatment and at various times after administration of 4 units of E-negative packed RBC. The cells were washed either with saline-BSA or with saline containing ex vivo antigens (saline-U5).[6] As shown in Table 2, IgG, IgM, and C3d could be demonstrated on the RBC in both instances, but the reactions due to IgG and IgM were more pronounced when the cells were washed with saline-U5. The strength of agglutination decreased with time (Table 2). No antibody could be demonstrated on the RBC after day 10. These findings indicated that the antibodies belonged to the IgG and IgM classes and were, at least partly, dependent on the presence of the drug and/or its metabolites.

Table 1. Patient's Serum, Day 0, Tested in the Absence of Nomifensine and Its Metabolites

			Titer of Agglutination With			
Tested Against RBC	Whole Serum		Serum Absorbed With RBC		Eluates Obtained From RBC	
	Undialyzed	Dialyzed	R_2R_2	rr	R_2R_2	rr
R_2R_2	8	8	—	4	16	—
R_1R_2	2	2	—	1*	4	—
rr	—	—	—	—	—	—
Rh_{null}	NT	NT	NT	NT	—	—

Key: —, negative; *, weak but stable reaction with undiluted serum, NT, not tested.

Isolation of antibodies by elution. Antibodies were eluted from the patient's RBC after washing with saline-BSA or saline-U5, respectively,[6] and were then tested against R_2R_2, rr, and Rh_{null} RBC in the presence or absence of ex vivo antigens (Table 3). Using day 0 eluates, drug-independent agglutination was observed only with R_2R_2 RBC. Metabolite-dependent reactions were found in the IAT predominantly with rr RBC. With day 6 eluates, only metabolite-dependent positivity in the IAT was detectable; it was clearly stronger with rr than with R_2R_2 and very weak with Rh_{null} RBC. After the patient had been hospitalized for 2 weeks, no antibody could be eluted from her red cells.

Immunoglobulin classes and hemolytic activity of antibodies in relation to drug dependency and Rh specificity. Whole serum from day 15 and immunoglobulin fractions (IgG, IgA, IgM) were tested concomitantly against R_2R_2, R_1R_2, rr, and when possible, Rh_{null} RBC in the presence of nomifensine, its metabolites (M1, M2, M3), and its ex vivo antigens. The results are summarized in Table 4. Two important observations were made. First, antibody activity was detectable in the presence of metabolites and/or ex vivo antigens (U5), but not in the presence of nomifensine, clearly indicating metabolite-specific antibodies. Second, at least two (possibly three) different metabolite-dependent antibodies were noticed: an IgG antibody exclusively reactive with ex vivo antigens exhibiting predominantly anti-e specificity and an agglutinating IgM antibody preferentially reactive with M2 without obvious Rh specificity. However, when the hemolytic activity of serum and immunoglobulin fractions was analyzed (Tables 4 and 5), it became evident that hemolysis was confined to IgM antibodies and occurred mainly with R_2R_2 RBC, possibly due to a third metabolite-specific antibody. The IgA fraction did not react with RBC.

DISCUSSION

The data clearly show that the hemolytic episode in the patient described here was the result of multiple nomifensine-induced antibodies. Like previous reports of nomifensine-dependent IHA,[1-6] the hemolysis was acute, intravascular, and remitted promptly after discontinuation of the drug. Renal failure, often seen in this syndrome,[6] was transient but required temporary hemodialysis.

From an immunologic point of view the most intriguing and as yet undescribed finding was the apparent Rh specificity of nomifensine-induced free as well as RBC-bound antibodies. This observation bears particular significance on our understanding of the underlying pathogenetic mechanism. Initially an anti-E was detectable in the serum and in eluates prepared from autologous RBC before transfusion. Its reactivity was clearly drug independent. The assumption that the antibody represented an ordinary alloantibody is very unlikely for the following reasons: the patient had until then never been transfused, the antibody disappeared quickly after withdrawal of the drug, and it could be eluted from autologous RBC although the patient had the Rh phenotype CCDee. We therefore favor the interpretation that the

Table 2. DAT on the Patient's Red Cells

		Reaction With Anti-			
Day	Therapy	IgG	IgA	IgM	C3d
0	None	2-3+/4+	–/–	1-2+/2+	2-3+/2-3+
1, 2	Blt† (4U), hemodialysis	NT	NT	NT	NT
3	Hemodialysis	2+/3-4+	–	–/1-2+	2+/2+
6	Hemodialysis	1+/2+	–	–/–	1-2+/1-2+
10, 15, 22	None	–/–	–	–/–	1+/1+

DAT was performed with cells washed either in saline containing 0.2% BSA or saline containing ex vivo antigen (U5) of nomifensine, respectively (–/–).

Key: Blt, blood transfusion; –, negative; 4+ > 3-4+ > 3+ > 2-3+ > 2+ > 1-2+ > 1+.

reactivity of this antibody appeared to be directed against an E-like antigen on human RBC, a situation resembling the pseudo–anti-E specificity described in patients with autoimmune hemolytic anemia.[8–10]

The antigenic determinants of the other antibodies, either bound to RBC or free in the serum, appeared to consist of compound structures related both to metabolites of nomifensine and RBC membranes. This phenomenon became particularly obvious upon separate analysis of serum and immunoglobulin fractions in the presence of nomifensine, its main metabolites, and its ex vivo antigens (presumably comprising additional as yet unidentified metabolites) against RBC with different Rh phenotypes. The IgG fraction was strongly reactive with rr, less with R_1R_2, and weak with R_2R_2 or Rh_{null} RBC in the presence of ex vivo antigen, but not in the presence of nomifensine or its known metabolites. This reaction pattern resembles again that seen with warm anti-e autoantibodies, which often react more strongly with e-positive than with e-negative RBC.[11] Experiments with the IgM fraction of the same serum sample yielded a more complex picture in that reactions were strong with all RBC in the presence of ex vivo antigen and M2, but weaker in the presence of M1 and M3, presumably reflecting cross-reactivity with M2.[6] On the other hand, E-positive cells were found to be more susceptible to complement-mediated lysis by the same antibody under identical conditions. This phenomenon remained inexplicable because absorption and elution of antibodies could be accomplished with rr as well as R_2R_2 RBC irrespective of whether the IgM fraction or whole serum was used (data not shown).

Although the in vitro reaction of these antibodies seemed to be dependent solely on the presence of metabolites or the drug (augmented or exclusive reactivity in the presence of ex vivo antigen), the antibodies remained attached to RBC in vivo even in the absence of the drug or its metabolites. This notion that the positive DAT is drug independent is supported by the fact that nomifensine is quickly metabolized in vivo[12] and that the antibodies remained recognizable on patient's RBC longer than six days after discontinuation of nomifensine and after transfusion of 4 units of packed RBC. It is therefore unlikely that residual metabolites were a prerequisite for the positive DAT. Indeed, a serum sample obtained from the patient ten hours after the last drug ingestion and a urine specimen of the following day did not seem to contain nomifensine or metabolites as indicated by their failure to be utilized as ex vivo antigens with known nomifensine-induced antibodies (sera 1 and 7[6]). How, then, can the enhanced positivity of the DAT and of the eluates in the presence of ex vivo antigen be explained? It is conceiv-

Table 3. Reactions of Eluates Prepared From the Patient's Red Cells

Patient's RBC		Titer of Eluted Antibodies Tested by Agglutination/IAT* against RBC with Rh Antigens					
		R_2R_2†		rr†		Rh_{null}†	
Day	Washed With	Absent	Present	Absent	Present	Absent	Present
0	saline-BSA	2/SA‡	2/SA	–/–	–/4	–/–	–/1
	saline-U5	NT	NT	NT	NT	NT	NT
6	saline-BSA	–/–	–/–	–/–	–/–	–/–	–/–
	saline-U5	2/SA	2/SA	–/–	–/16	–/–	–/2
10	saline-BSA	–/–	–/–	–/–	–/–	–/–	–/–
	saline-U5	–/–	–/2	–/–	–/16	–/–	–/1
15	saline-BSA	–/–	–/–	–/–	–/–	–/–	–/–
	saline-U5	–/–	–/–	–/–	–/–	–/–	–/–

Before elution the cells were washed in the presence (saline-U5) or absence of ex vivo antigens (saline-BSA) and tested as indicated.

*Using polyspecific antiglobulin serum.

†In the presence or absence of ex vivo antigen (U5)

‡Strong agglutination unchanged after washing.

Table 4. Patient's Serum and Serum Fractions Tested in the Presence of Nomifensine and its Metabolites

Antibodies Present on Day 15 in	RBC Tested	Titer of Antibodies in Agglutination/IAT* in the Presence of					
		N	M1	M2	M3	U5	U0
Whole serum	rr	–/–	8/4	8/8	4/–	32/256	–/–
	R_1R_2	–/–	8/8	8/32	4/1	64/128	–/–
	R_2R_2	–/–	8/8	8/32	8/4	64/128	–/–
IgG fraction†	rr	–/–	–/–	–/–	–/–	–/32	–/–
	R_1R_2	–/–	–/–	–/–	–/–	–/4	–/–
	R_2R_2	–/–	–/–	–/–	–/–	–/2	–/–
	Rh_{null}	–/–	–/–	–/–	–/–	–/2	–/–
IgM fraction†	rr	–/–	2/2	8/8	–/2	8/8	–/–
	R_1R_2	–/–	2/2	8/8	–/2	8/8	–/–
	R_2R_2	–/–	2/2	8/32‡	–/2	16/32‡	–/–
	Rh_{null}	–/–	NT	NT	NT	8/8	–/–

Tests were conducted on nomifensine (N); its metabolites M1, M2, and M3; its ex vivo antigen (U5); and drug-free urine (U0, control).

*With polyvalent antiglobulin serum; fresh AB serum (complement) was added to each tube before washing.

†Immunoglobulin fractions were absorbed six times with A_1 RBC in order to remove anti-A, which would have reacted with group A Rh_{null} RBC.

‡Hemolysis was observed.

Table 5. Hemolytic Activity of Metabolite-Dependent Antibodies

	Red Cell Rh Phenotype			
	R_2R_2	R_2R_2	rr	rr
Whole serum*	40†	45	8	16
IgM fraction	12	10	—	—
IgG fraction	—	—	—	—

*Day 15 serum and fractions.

†Percent hemolysis in the presence of ex vivo antigen (U5) and complement

able that these antibodies recognize variable structural sites on the RBC surface to which they attach rather loosely in the absence, but more strongly in the presence of the drug or its metabolites. Another possible explanation for the transiently positive DAT is a simultaneous coating of RBC with residual metabolites and antibodies. In either possibility antibody binding involves defined structures on the RBC surface. This is supported by the fact that a drug-related antibody reacts only with one type of blood cell, ie, with platelets and not with RBC.[13,14]

Considering all these findings, there is now suggestive evidence that target cells in patients with drug-induced immune cytopenias of the so-called immune-complex type do not act as innocent bystanders, but rather serve as "carrier" surfaces. Although conjugation of drugs and cells in vitro does not appear stable, it might still suffice to render this complex immunogenic in some patients. Hence, it is not surprising that the antibodies in such patients, like those in animals immunized with hapten-protein conjugates, will be quite heterogeneous with regard to their binding sites on the conjugates.[15,16] A similar suggestion related to drug-induced immune cytopenias has recently been made by Habibi[17] and is further corroborated by a number of reports[18–23] that indicate the presence of receptors for certain drug-dependent antibodies on target blood cells.

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REFERENCES

1 Habibi B, Cartron JP, Bretagne M, Rouger P, Salmon C· Anti-nomifensine antibody causing immune hemolytic anemia and renal failure. Vox Sang 40:79, 1981

2. Eckstein R, Riess H, Sauer H, Mempel W: Immunhämolytische Anämien mit Nierenversagen nach Nomifensineinnahme. Klin Wochenschr 59:567, 1981

3 Lyloff K, Jersild C, Bacher T, Slot O: Massive intravascular haemolysis during treatment with nomifensine. Lancet 2 41, 1982 (letter)

4. Salama A, Mueller-Eckhardt C, Kissel K, Pralle H, Seeger W: Ex vivo antigen preparation for the serological detection of drug-dependent antibodies in immune haemolytic anaemias. Br J Haematol 58:525, 1984

5. Sokol RJ, Hewitt S, Booker DJ, Stamps R, Taylor M: Fatal immune haemolysis associated with nomifensine. Br Med J 291:311, 1985

6 Salama A, Mueller-Eckhardt C: The role of metabolite-specific antibodies in nomifensine-dependent immune hemolytic anemia New Engl J Med 313:469, 1985

7. Petz LD, Branch DR: Drug-induced immune hemolytic anemia, in Chaplin H Jr (ed). Immune Hemolytic Anemias. New York, Churchill Livingstone, 1985, p 47

8 Issitt PD, Zellner DC, Rolih SD, Duckett JB· Autoantibodies mimicking alloantibodies Transfusion 17:531, 1977

9 Issitt PD, Pavone BG: Critical re-examination of the specificity of auto-anti-Rh antibodies in patients with a positive direct antiglobulin test Br J Haematol 38.63, 1978

10 Rand BP, Olson JD, Garratty G, Petz LD: Coombs' negative immune hemolytic anemia with anti-E occurring in the red blood cell eluate of an E-negative patient. Transfusion 18:174, 1978

11. Petz LD, Garratty G: Acquired Immune Hemolytic Anemias. New York, Churchill Livingstone, 1980, p 232

12 Saletu B, Grünberger J, Linzmayer L, Taeuber K. The pharmacokinetics of nomifensine: Comparison of pharmacokinetics and pharmacodynamics using computer pharmaco-EEG. Int Pharmacopsychiatry 17:43, 1982 (suppl 1)

13. Shulman NR A mechanism of cell destruction in individuals sensitized to foreign antigens and its implications in auto-immunity. Combined Clinical Staff Conference at the National Institutes of Health. Ann of Intern Med 60.506, 1964

14. Petz LD, Fudenberg HH: Immunologic mechanisms in drug-induced cytopenias, Brown EB (ed)· in Progress in Hematology, vol 9. Orlando, FL, Grune & Stratton, 1975, p 185

15. Benacerraf B, Levine BB: Immunological specificity of delayed and immediate hypersensitivity reactions J Exp Med 115·1023, 1962

16 Silverstein AM, Gell PGH· Delayed hypersensitivity to hapten-protein conjugates II. Anti-hapten specificity and heterogeneity of the delayed response J Exp Med 115·1053, 1962

17. Habibi B Drug induced red blood cell autoantibodies co-developed with drug specific antibodies causing haemolytic anaemias Br J Haematol 61 139, 1985

18. Letona JML, Barbolla L, Frieyro E, Bouza E, Gilsanz F, Fernández MN: Immune haemolytic anaemia and renal failure induced by streptomycin. Br J Haematol 35.561, 1977

19. Claas FHJ, Langerak J, de Beer LL, van Rood JJ: Drug-induced antibodies Interaction of the drug with a polymorphic platelet-antigen Tissue Antigens 17:64, 1981

20. Duran-Suarez JR, Vega-Martin C, Argelagues E, Massuet L, Ribera A, Triginer J Red cell I antigen as immune complex receptor in drug-induced hemolytic anemias Vox Sang 41:313, 1981

21. Kunicki TJ, Christie DJ, Aster RH· The human platelet receptor(s) for quinine/quinidine-dependent antibodies. Blood Cells 9:293, 1983

22. Sosler SD, Behzad O, Garratty G, Lee CL, Postaway N, Khomo O: Acute hemolytic anemia associated with a chlorpropamide-induced apparent auto-anti-Jk^a. Transfusion 24:206, 1984

23. Jordan JV, Smith ME, Reid ME, Jones CE, Kautz CA, Shulman NR A tolmetin-dependent antibody causing severe intravascular hemolysis binds to erythrocyte band 3 and requires only the $F(ab)_2$ domain to react Blood 66:104, 1985

L-Triiodothyronine Augments Erythropoietic Growth Factor Release From Peripheral Blood and Bone Marrow Leukocytes

By Nicholas Dainiak, David Sutter, and Sandra Kreczko

To investigate cellular mechanisms involved in thyroid hormone stimulation of erythropoiesis, we studied the response of erythroid burst-forming unit (BFU-E) proliferation to L-triiodothyronine (L-T3) in a serum-free culture system. When added directly to culture, L-T3 stimulates erythroid burst formation by normal human bone marrow cells. In contrast, granulocyte-macrophage colony formation is unaffected. Enhancement of erythroid burst formation by L-T3 required the presence of nylon wool adherent and/or B-4 antigen-positive light-density marrow populations. Addition of other erythropoietic factors including platelet-derived growth factor and insulinlike growth factor II did not abrogate this apparent cellular requirement. Pulse exposure of marrow and peripheral blood mononuclear cells (> 95% lymphocytes) to L-T3 accelerates the release of a soluble factor that augments BFU-E proliferation into serum-free liquid culture medium. Time-course studies show that this factor appears in conditioned medium (CM) coincidentally with erythroid burst-promoting activity (BPA). Furthermore, incubation of CM with an antibody known to react with and adsorb BPA from solution removes the inducible mitogen. Biochemical analysis of CM prepared from unexposed and L-T3 pulse-exposed cells indicates that the rate of protein appearance is accelerated by L-T3 in a fashion that immediately precedes growth factor release and that several polypeptides are quantitatively increased. We conclude that unlike erythropoietin, which is mitogenic for progenitor cells directly, L-T3 enhances BFU-E proliferation indirectly by augmenting the release of soluble BPA-like molecules from accessory cells in culture.

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PROLIFERATION of erythroid progenitor cells in tissue culture is dependent on the presence of tissue-specific growth factors that include erythropoietin and burst-promoting activity (BPA). In addition, classic and paracrine hormones whose mitogenic actions extend beyond hematopoietic tissue influence the formation of colonies derived from these cells.[1-3] We have previously shown that one group of public hormones, the thyroid hormones, augments human erythroid colony formation and that a primitive progenitor known as the burst-forming unit–erythroid (BFU-E) is particularly sensitive to the effects of L-triiodothyronine (L-T3).[4] Here, we investigate cellular mechanisms involved in the expression of erythropoietic activity by L-T3 in vitro by asking whether L-T3 has a direct effect on BFU-E progenitors or whether its action is mediated via accessory hematopoietic cells.

The role of BPA in supporting early erythroid differentiation and proliferation has been previously determined.[5,6] This factor is spontaneously released into liquid culture medium from light-density mononuclear cells obtained from peripheral blood and bone marrow.[7-10] To test the hypothesis that L-T3 augments growth factor production by these cells, we have measured the release of protein and erythropoietic growth factor(s) into serum-free medium conditioned by human circulating and marrow leukocytes that were pulse exposed to L-T3. We have found that L-T3 accelerates the rate of release of a soluble factor that stimulates erythroid burst formation by human marrow cells in serum-free culture. Preliminary analysis has revealed that the rate of protein appearance in membrane vesicle–free supernatants of leukocyte-conditioned medium is augmented by L-T3 and that several polypeptides are selectively induced. Soluble L-T3–induced growth factors were absorbed from conditioned medium (CM) with an antibody that recognizes BPA molecules. Stimulatory activity expressed by L-T3 added directly to culture was found to require the presence of nylon-adherent and B-4–positive marrow cells. We suggest that thyroid hormones support BFU-E proliferation by enhancing the production of BPA-like molecules in accessory cells in the bone marrow.

MATERIALS AND METHODS

Treatment with L-T3. Peripheral blood and bone marrow were collected from 30 healthy, paid volunteers with informed consent and approval of an institutional review board. Samples were mixed with alpha-medium (Gibco 320-2561, GIBCO, Grand Island, NY) plus 20 U/mL preservative-free heparin and separated over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Light-density mononuclear cells were removed, mixed with a carbonyl iron suspension (Technicon Instruments Corp, Tarrytown, NY), and separated over Ficoll-Hypaque again. Interface cells that were composed of >95% lymphocytes were split into equal aliquots and suspended to a density of 5×10^6/mL in serum-free alpha-medium with or without L-T3 A stock solution of 3,3',5-triiodothyronine (Sigma Chemical Co, St Louis), 10^{-3} mol/L, was prepared in 95% ethanol and added to alpha-medium to obtain final concentrations of 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} mol/L. Cells in each aliquot (L-T3 exposed and unexposed) were incubated in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) for 15 to 120 min at 37 °C in humidified air, pelleted by centrifugation at 280 g for 20 minutes, and washed three times. Cell viability assessed by trypan blue exclusion and by staining with ethidium bromide and acridine orange was >99%. In some cases, L-T3 was added directly to the bone marrow culture at final concentrations indicated in Table 1 and the Figure legends.

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Table 1. Stimulation of BFU-E Proliferation by the Addition of L-T3 Directly to Adherent and Nonadherent Marrow Cell Cultures

Marrow Cell Fraction	L-T3 (10^{-8} mol/L)	No Bursts/ 2×10^5 Cells
Experiment 1		
Unseparated	−	31 ± 4
Nylon adherent	−	8 ± 2
Nylon nonadherent	−	15 ± 3
Adherent + nonadherent	−	28 ± 2
Unseparated	+	57 ± 8
Nylon adherent	+	15 ± 2
Nylon nonadherent	+	14 ± 3
Adherent + nonadherent	+	46 ± 7
Experiment 2		
Unseparated	−	24 ± 2
B4-negative (nonadherent)	−	8 ± 1
B4-positive (adherent)	−	0 ± 0
Adherent + nonadherent	−	16 ± 3
Unseparated	+	36 ± 3
B4-negative (nonadherent)	+	12 ± 2
B4-positive (adherent)	+	not tested
Adherent + nonadherent	+	25 ± 3
Experiment 3		
Unseparated	−	19 ± 3
E rosette-negative	−	15 ± 2
Unseparated	+	30 ± 5
E rosette-negative	+	27 ± 4

Ficoll-Hypaque-separated marrow cells were separated by adherence to nylon (experiment 1) or Ig-coated sheep erythrocytes (experiment 2) and cultured in fibrin clots containing 2.0 IU/mL sheep erythropoietin They were depleted of sheep erythrocyte-rosetting populations (experiment 3) by incubation with a cytotoxic monoclonal antibody plus complement (see Materials and Methods). Mean ± SEM bursts in each of four clots are displayed Similar results were obtained in two additional cultures of separated marrow cells.

Preparation of CM Fresh and preincubated, L-T3 pulse-exposed and unexposed cells were immediately suspended to a density of 5×10^6/mL in tissue culture flasks in alpha-medium with L-glutamine, streptomycin, and penicillin (GIBCO). The cells were incubated at 37 °C and 4% CO_2, pelleted, and resuspended sequentially every 15 to 120 minutes for up to 72 hours The CM was harvested and tested in culture for mitogenic activity, and protein content and composition were determined Radioimmunoassay for thyroxine and triiodothyronine of CM prepared from cells exposed to 10^{-9} mol/L L-T3 for 90 minutes demonstrated undetectable levels of thyroid hormone.

In several experiments, the CM was subjected to centrifugation at 40,000 g for 60 to 120 minutes. The resulting supernatants and plasma membrane–derived vesicle-rich pellets[10] were assayed for protein and BPA contents.

Assay and unitage for erythroid mitogens. Approximately 0.5 mL of well-spiculated bone marrow was aspirated from the posterior iliac crest of 25 healthy, paid donors Informed consent was obtained for each donation, and approval of an institutional review board was granted prior to initiation of this study. The cells were placed in Eagle's minimal essential medium (MEM) (GIBCO) containing heparin and separated over Ficoll-Hypaque. Mononuclear cells appearing at the interface were separated, washed three times in alpha-medium, and cultured at densities of 0.5, 1, 2, 3, 4, and 5 × 10^5/mL In some cases, light-density cells were first resuspended at 5×10^6/mL, and passed over a 3-mL nylon fiber column.[7,10] Morphologically, light-density, nylon-adherent cells were composed primarily of lymphocytes (mean ± SEM, 55% ± 9%) and monocytes (18% ± 3%), while light-density, nylon-nonadherent cells consisted chiefly of lymphocytes (63% ± 11%) and early and mature myeloid elements (14% ± 6%). Fewer than 5% of the nucleated cells in either fraction were benzidine-positive Fewer than 3% of the nylon-adherent lymphocytes and greater than 95% of the nylon-nonadherent lymphocytes rosetted with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes.

In other experiments, 6×10^5 light-density cells were coated with mouse anti-B4 antibody (100 μg/mL, 30 minutes, 4 °C) obtained from Dr Albert Najman, Faculte de Medecine Saint-Antoine, Paris The antigen recognized by this antibody is B cell specific and distinct from other known B cell antigens.[11] It is present on both resting and mitogen-stimulated B cells and is increased with B cell activation It is absent from resting and activated T cells and myeloid cells. Next, purified sheep antimouse IgG (1 mg/mL) was coupled to sheep erythrocytes by the chromium chloride method[12] using equal volumes of washed packed erythrocytes, 0.1% (wt/vol) chromium chloride, and antibody (five minutes, 23 °C). Antibody-coated marrow cells were then incubated with a 2% solution of sheep erythrocytes for five minutes at 4 °C, and the mixture was centrifuged over Ficoll-Hypaque. Cells at the interface (nonadherent) and in the pellet (adherent to erythrocytes from which they were freed by lysis in 0.17 mol/L NH_4Cl) were separated[13] and added to culture. Nonadherent populations consisted of 1% to 2% surface Ig–bearing cells, and adherent populations consisted of 90% to 96% surface Ig–positive cells by immunofluorescence staining In addition, tests with T cell–depleted human bone marrow were performed with a cytotoxic murine monoclonal anti–T cell antibody[13] prepared by Dr A. Bernard (Institut Gustave, Roussy, France). Briefly, 2×10^7 light-density marrow cells in 1 0 mL alpha-medium were incubated with 200 μg/mL antibody plus an equal volume of rabbit complement (Calbiochem-Behring Corp, La Jolla, CA) for 30 minutes at 37 °C. Fewer than 1% of the cells surviving the incubation rosetted with AET-treated sheep erythrocytes.

Serum-free marrow cultures were established in 0.8% methylcellulose or fibrin clots as described previously.[14] They contained Iscove's modified Dulbecco's medium (IMDM) (GIBCO), purified human serum albumin,[14] transferrin, ferric chloride, and 0.0, 0.1, 0 5, 1.0, 2.0, or 4.0 U/mL sheep step III erythropoietin (Connaught Laboratories, Toronto), human urinary erythropoietin (70,000 U/mg, a generous gift of Dr Eugene Goldwasser, University of Chicago), or recombinant human erythropoietin (70,000 to 80,000 U/mg, Amgen Biologicals, Thousand Oaks, CA). Test plates contained 10% (vol/vol) L-T3, CM, or NCTC-109 (Microbiological Associates, Bethesda, MD), unless otherwise indicated. Cultures were maintained for 12 to 14 days at 37 °C and 4% CO_2 and scored for orange bursts (BFU-E–derived colonies) with a grid under an inverted microscope or (in the case of clots) under 100 × magnification. Bursts consisted of ≥50 (and often as many as 500) cells that were benzidine-positive Cloning efficiency varied from 15 to 60 bursts/10^5 light-density cells plated.

Mitogenic activity was determined in CM prepared from preincubated, L-T3–exposed cells by comparing burst formation in cultures plated with this CM to that with CM prepared from cells preincubated with alpha-medium alone One unit of BPA is operationally defined as equivalent to 100% or twofold stimulation above burst formation in cultures containing 10% (vol/vol) NCTC-109. The amount of BPA present in each CM was corrected for the volume of CM added to culture (0 2 mL or 0.1 mL for methylcellulose and fibrin clot cultures, respectively) and is expressed per a 1-mL volume. Rates of BPA release were adjusted for time of incubation and are expressed as units per milliliter per hour.

Interactions of L-T3 with other erythroid mitogens Because several circulating factors are known to augment erythroid progenitor cell proliferation,[15,16] we considered whether one or more serum mitogens other than erythropoietin may serve as potential cofactors for an L-T3 effect Serum-free cultures of adherent and nonadherent marrow cells obtained after panning with the anti-B4 antibody were established. L-T3 (10^{-9} mol/L) was added in the presence and absence of platelet-derived growth factor (PDGF) and/or insulinlike growth factor II (IGF-II) at concentrations that will be indicated in Fig 4. Electrophoretically pure porcine PDGF was obtained from Bethesda Research Laboratories (Gaithersburg, MD) This material has an apparent molecular weight (mol wt) of 38,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gels and is biochemically similar to human PDGF.[17] Dr M.P. Czech (University of Massachusetts, Worcester) kindly provided IGF-II purified from serum-free medium conditioned by buffalo rat liver cells (BRL-3A).[18] Samples were dissolved in 0.005 *N* HCl and stored at −20 °C until use. A final volume of 100 μL of growth factor or NCTC-109 was added to culture

Assay for granulocyte-macrophage colony-stimulating activity (GM-CSA) To determine whether L-T3 induced the release of factors that are mitogenic for the granulocyte/macrophage progenitor (CFU-GM), we assayed media conditioned by L-T3–exposed and unexposed cells plus 1% phytohemagglutinin in human peripheral blood culture. Briefly, the method of Pike and Robinson[19] was modified by the addition of fivefold serum substitute[14] or 20% fetal calf serum that was adsorbed with activated charcoal to remove thyroid hormones prior to culture.[3] Human blood mononuclear cells served as a standard source of GM-CSA.[20] Results were compared to cultures containing CM, NCTC-109, or L-T3 (10^{-9} mol/L) added directly to culture.

Biochemical characterization of L-T3–induced components CM and medium fractions were analyzed for total protein by the Bio-Rad microassay (Bio-Rad Laboratories, Richmond, CA) and by the method of Lowry et al,[21] respectively, using bovine serum albumin (BSA) as the standard.

For gel electrophoresis, 10 to 30 mL of supernatants of CM was extensively dialyzed, lyophylized, resuspended to 50 μL in distilled water, and electrophoresed in one-dimensional 3.5% to 18% SDS/polyacrylamide gels, according to Laemmli.[22] Membrane vesicle–containing pellets were resuspended in Na-phosphate and similarly prepared. Gels were stained first with silver and then with Coomassie brilliant blue (CB) according to Dzandu et al.[23] Sialoglycoproteins and lipids (yellow Ag-stained bands) and proteins (blue CB-stained bands) were visualized by this method

Immunologic characterization of L-T3–induced mitogens To investigate the relatedness of BPA and L-T3–induced mitogens, we employed a polyclonal antimembrane antibody preparation that is known to cross-react with components of CM supernatants and to neutralize BPA in vitro.[20] Membrane vesicle–free supernatants (200 μL) were incubated at 25 °C for 45 minutes with 0, 100, or 200 μg of normal rabbit IgG or rabbit antihuman lymphocyte plasma membrane IgG that was purified on diethylaminoethanol cellulose. Antigen-antibody complexes and free IgG were removed by incubation for 30 minutes at 25 °C with an excess amount of a 10% staphylococcal protein A solution and centrifugation at 1,200 g for 60 minutes. Absorbed supernatants were added to culture, and results were compared to those obtained with assays of unabsorbed supernatants.

Statistical analysis. Mean ± 1 SEM bursts appearing in triplicate 2.0-mL methylcellulose cultures and quadruplicate 125-μL fibrin clots were determined. Data sets were compared by the two-sample ranks test of Wilcoxon-White according to Goldstein.[24]

RESULTS

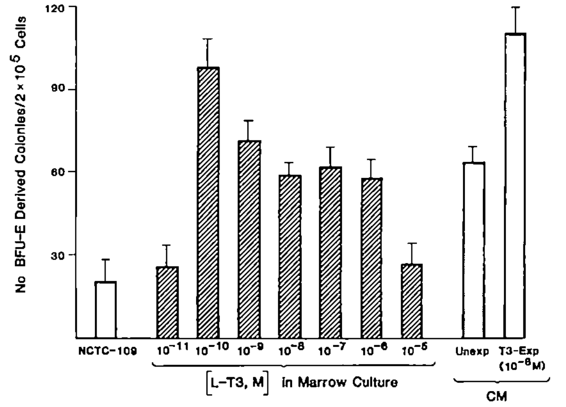

Fig 1. Stimulation of burst formation by L-T3. Mean ± SEM bursts are displayed for fibrin clot cultures plated with added L-T3 (hatched bars) or with CM prepared from L-T3-exposed or unexposed circulating mononuclear cells (stippled bars). Cultures contained 2.0 IU/mL human erythropoietin, IMDM, iron-saturated transferrin, purified human serum albumin, thrombin, and fibrinogen.[12] Burst formation in cultures with 10^{-10} to 10^{-6} mol/L L-T3 was greater than that in cultures containing NCTC-109 (open bar). It was also greater in cultures with CM prepared from L-T3–exposed cells than in those with either CM from unexposed cells or a similar amount (10^{-8} mol/L) of L-T3 alone added directly to culture ($P < .05$ for each).

Burst stimulation by L-T3 and CM. As shown in Fig 1, relative to burst formation in NCTC-109–containing cultures, bursts were increased in cultures supplemented directly with L-T3 ($P < .05$). The dose-response curve is biphasic, with an optimal final L-T3 concentration of 10^{-10} mol/L. Stimulation was similar over the full range of the erythropoietin concentration tested, and no bursts formed in the absence of erythropoietin. In contrast, burst enhancement was greatest in cultures with high marrow cell–seeding densities and was absent as the limiting dilution was approached (see Fig 2). Stimulation was absent in cultures of

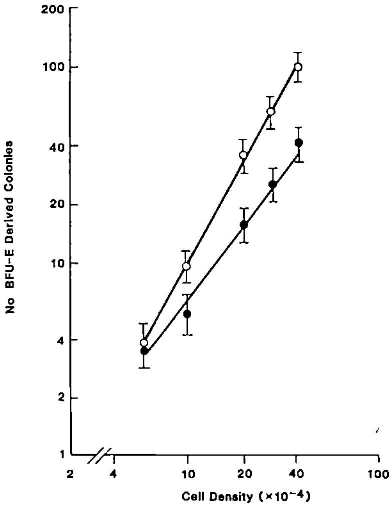

Fig 2. L-T3 activity as a function of marrow-seeding concentration. Mean ± SEM bursts per culture are shown for methylcellulose cultures plated with (○) and without (●) the direct addition of 10^{-9} mol/L L-T3 to 2.0 IU/mL human erythropoietin. Stimulation by L-T3 is greatest at the marrow cell density of 4 × 10^5/mL ($P < .05$) and absent at the concentration of 6 × 10^4/mL ($P > .10$).

nylon-nonadherent bone marrow cells, an effect that was reversible by adding back the adherent cells (see Table 1). Similar findings were made in cultures of marrow cells enriched in and depleted of B lymphocytes using a monoclonal antibody. In contrast, removal of T lymphocytes from light-density marrow populations did not alter the stimulatory effects of L-T3 (see Table 1).

When CM prepared from L-T3–unexposed cells was added to culture, burst formation was augmented in a characteristic fashion (see Fig 1). However, the addition of CM harvested after incubation with cells that were previously pulse exposed to L-T3 resulted in a further increment in burst number (relative to CM prepared from L-T3–unexposed cells, $P < .05$). A similar result was observed in cultures containing CM prepared from marrow cells that were pulse exposed to L-T3 (data not shown). The minimum time of pulse exposure for an L-T3 effect was 90 minutes, and pulse exposures to L-T3 of longer than 90 minutes did not result in greater stimulation by CM. Figure 3 shows that a biphasic dose-response relationship exists also for the release of BPA-like factors from circulating and marrow leukocytes into CM. Together, the data indicate that augmentation of BFU-E proliferation by the direct addition of L-T3 to culture requires the presence of accessory marrow cells and that L-T3 promotes the release of one or more erythropoietic growth factors from circulating and marrow mononuclear cells.

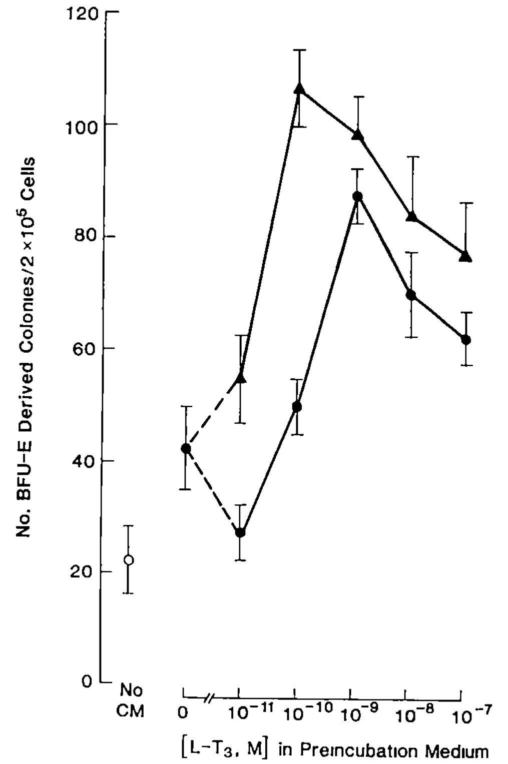

Fig 3 Effects of preincubation of leukocytes with L-T3 on growth factor production. Mean ± SEM bursts are shown for methylcellulose cultures containing 10% (vol/vol) NCTC-109 (open circle), CM prepared from L-T3–unexposed cells (zero), and CM prepared from cells pulse exposed to L-T3 90 minutes at the indicated concentrations. Cultures contained 2.0 IU/mL human erythropoietin. Circles, CM prepared from circulating cells; triangles, CM prepared from light-density marrow cells.

Interactions of L-T3, PDGF, and IGF-II. Since it is possible that the inability of L-T3 to augment burst formation in cultures of B lymphocyte–depleted marrow cells is due to the lack of a necessary cofactor, we examined the effects of L-T3 in cultures containing other serum mitogens. As shown in Fig 4, we observed that L-T3 activity in B cell–containing (unseparated) marrow cultures was additive with that of PDGF and IFG-II. However, L-T3 did not augment BFU-E proliferation in cultures of B cell–depleted bone marrow regardless of whether PDGF and/or IGF-II were also present. The latter finding suggests that neither PDGF nor IGF-II exerts permissive activity for L-T3 action on erythroid burst formation. However, the possibility that other serum molecules may serve as cofactors has not been excluded.

Kinetics of L-T3–induced growth factor release. To quantify L-T3 effects on growth factor production, we measured cumulative amounts of BPA and/or BPA-like factors released by cells that were sequentially pelleted and resuspended every 30 to 60 minutes for four hours and after a 24-hour incubation in fresh alpha-medium. The amount of BPA released into CM from L-T3–exposed cells was approximately twice that released from unexposed cells ($P < .05$). L-T3 enhancement of cumulative release was maximal at four hours (from 12 ± 3 to 31 ± 3 U/mL for medium conditioned by unexposed and exposed cells, respectively)

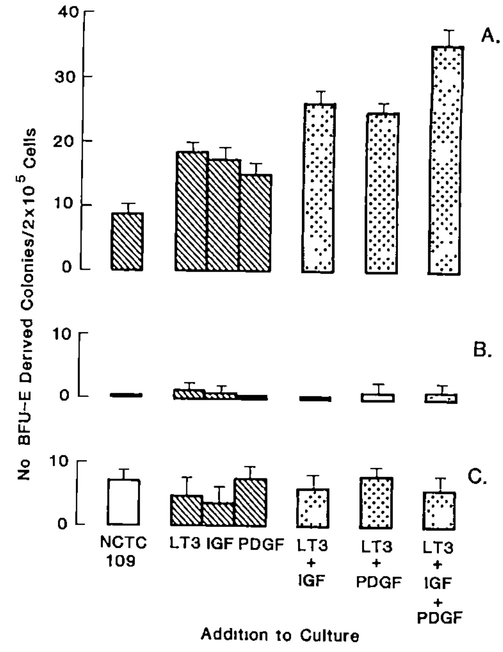

Fig 4. Influence of PDGF and IGF-II on L-T3 activity. Mean ± SEM bursts/2 × 10^5 cells are shown for cultures of unseparated (A), B4-negative (B), and B4-positive (C) marrow populations obtained after indirect panning (see Materials and Methods). Cultures contained 2.0 IU/mL human erythropoietin plus 10% (vol/vol) NCTC-109, L-T3 (10^{-9} mol/L), IGF-II (10 ng/mL), and/or PDGF (10 ng/mL).

and enhancement declined by 24 hours (from 18 ± 3 to 33 ± 2 U/mL, respectively).

Next, we determined the effects of preincubation with L-T3 on rate of growth factor release into CM. As shown in Fig 5, L-T3 transiently accelerates the rate of release of growth-promoting molecules from circulating leukocytes. Peak release (13 ± 2 U/mL/h) occurs at three hours of incubation, and rates of release are similar to control values by 24 hours (7 ± 2 *v* 8 ± 2 U/mL/h, respectively). Therefore, although the total amount of BPA released from L-T3–exposed cells after 24 hours (and also after 48 and 72 hours, not shown) is enhanced relative to that released from unexposed cells, rates of release are virtually equivalent by 24 hours of incubation.

L-T3 activity on soluble v *membrane vesicular BPA release.* We have previously shown that BPA is expressed by both soluble and pelletable, leukocyte plasma membrane vesicle–rich subfractions of CM.[10] Since L-T3 might exert a differential effect on the growth-promoting activities associated with each of these physically separable fractions, CM prepared from L-T3–exposed and unexposed leukocytes was fractionated and assayed in culture. Figure 6 shows that approximately half of assayable BPA present in CM is soluble and that L-T3–induced augmentation of growth-promoting activity is fully recovered in the soluble fraction. Moreover, the effects of L-T3 preexposure are most pronounced at suboptimal CM supernatant concentrations (10% and 20%, vol/vol, CM resulted in 3.1- and 2.0-fold stimulations of BPA release relative to unexposed cells, respectively) while a high concentration (30%) of CM showed no difference in BPA release. The data suggest that L-T3 induces the release of cytosolic or soluble membrane–derived molecules that appear to have BPA or BPA-like biologic activity in culture.

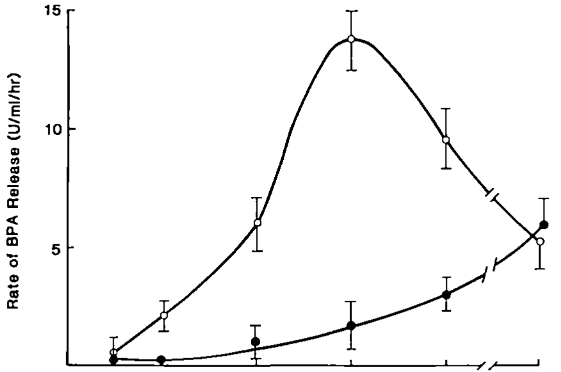

Fig 5. Influence of L-T3 on the rate of release of BPA and/or BPA-like molecules. Mean ± SEM U/mL/h are shown for two studies. CM preparation and marrow culture were performed as described in the legend to Fig 4. Cultures with NCTC-109 alone contained 26 ± 3 bursts/2 × 10^6 cells. Rates of release were calculated by adjusting for the time of incubation. L-T3 markedly augments the rate of BPA released within the first four hours of incubation ($P > .10$). Open circles, BPA in CM prepared from L-T3–exposed cells; closed circles, in CM prepared from unexposed cells.

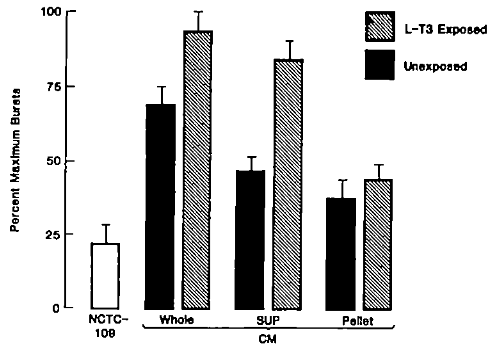

Fig 6. Effects of L-T3 on growth factors expressed by CM fractions. Whole CM prepared from circulating leukocytes that were preincubated with and without 10^{-9} mol/L L-T3 for 90 minutes was separated into supernatants (SUP) and membrane vesicle–containing pellets by high-speed centrifugation. Pellets were resuspended to the starting volume in NCTC-109. Mean ± SEM of bursts are shown for methylcellulose cultures containing 2.0 IU/mL human erythropoietin and 10% (vol/vol) whole CM, CM fractions, or NCTC-109. Cultures with 100% proliferation contained 115 bursts/2 × 10^6 marrow cells. Similar results were obtained in tests of subfractions of bone marrow cell CM.

Characterization of L-T3–induced cellular factors. To characterize effects of L-T3 on the composition of cellular components released during incubation, we measured the total protein contents of CM prepared from exposed and unexposed cells. Both cumulative protein release and rate of protein release were augmented by pulse exposure to L-T3 (Fig 7). Acceleration of the rate of protein release from 23 to 58 μg/mL/h occurred within two hours of incubation and rapidly decreased over time. Cumulative protein release by 24 hours was nearly twofold higher in CM prepared from L-T3–exposed cells (93 *v* 57 μg/mL). Cell viabilities after 24, 48, and 72 hours of incubation in serum-free media were 99%, 96%, and 87%, respectively. Longer incubations in serum-free medium resulted in excessive cell death (>15%). When analyzed on SDS/polyacrylamide gels, CM supernatant from L-T3–exposed cells contained increased amounts of glycoproteins and proteins with a mol wt approximate of 30 and 70 kilodaltons, respectively, relative to those present in supernatant prepared for unexposed cells. In contrast, no obvious differences were observed in the respective CM pellets (data not shown).

Next, we asked whether erythroid BPA might be related immunologically to the erythroid growth factor(s) induced by L-T3. Antimembrane IgG known to react with and neutralize soluble BPA was used to adsorb growth factors from supernatants of CM prepared from L-T3 pulse-exposed cells. Figure 8 shows that erythroid mitogens were removed from CM supernatant by incubation with IgG, followed by the addition of an excess amount of staphylococcal protein A and removal of the solid phase by centrifugation. In contrast, incubation with staphylococcal protein A alone or normal rabbit IgG plus staphylococcal protein A had no effect on the capacity of CM supernatant to support burst formation (Fig 8). The data raise the possibility that L-T3–induced factors and BPA that is spontaneously released from mononuclear cells may share antigenic determinants.

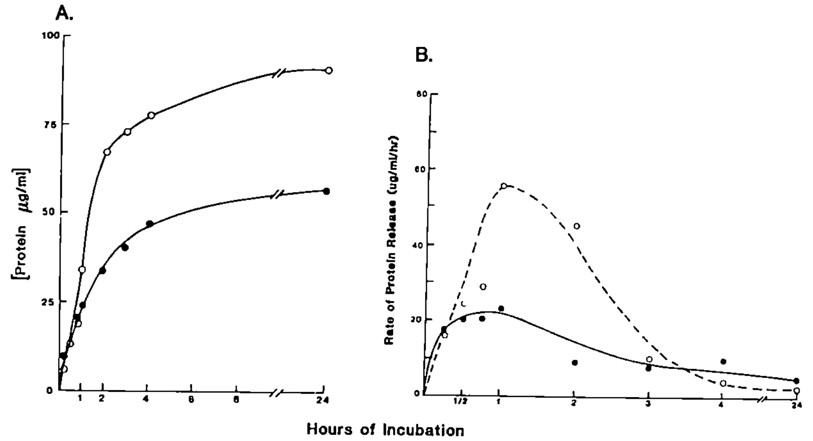

Fig 7. Effects of L-T3 on protein release. (A) Cumulative protein release into CM from circulating leukocytes that were preincubated with (O) and without (●) 10^{-9} mol/L L-T3 for 90 minutes Cells were washed and sequentially resuspended and pelleted every 15 to 60 minutes for four hours and after 24 hours. (B) Rates of protein release from L-T3-exposed (O) and unexposed (●) circulating leukocytes. The CM was prepared and sequentially harvested as described before.

Influence of L-T3 on CFU-GM proliferation. GM-CSA is known to be present in media conditioned by a variety of cell types including circulating light-density cells.[25-27] To investigate whether L-T3 augments the production of GM-CSA-like growth factors in a fashion analogous to induction of BPA-like molecule release, we assayed CM prepared from L-T3-exposed and unexposed cells for GM-CSA. Table 2 shows that while GM-CSA is present in our CM preparation, preexposure to L-T3 has no apparent enhancing effect on the expression of this activity in medium conditioned by such cells. Furthermore, addition of L-T3 to serum-free culture likewise does not augment CFU-GM proliferation (Table 2).

DISCUSSION

It is well known that clinical hypothyroidism is often complicated by reductions in red cell mass and total blood volume[28,29] and by normochromic, normocytic anemia.[30,31] The observation that anemia may be corrected by thyroxine alone[32] strongly suggests that it may be a consequence of hypothyroidism per se. To explain such in vivo findings, investigations have been undertaken that underscore the importance of thyroid hormones in the maintenance of adequate erythropoietin production[33-35] and in the direct stimulation of erythroid progenitor cells.[4,36-38] In this communication, we characterize a hematopoietic target cell population of thyroid hormones by utilizing a biochemically defined human bone marrow culture system while avoiding contamination with unknown quantities of polypeptide hormones, BPA, and extraneous growth factors[3] Since bovine and fetal calf sera contain biologically active L-T3 in concentrations of up to several hundred nanograms per milliliter or 10^{-8} mol/L, assays for L-T3-dependent growth effects are simplified under serum-free conditions.

Several observations suggest that L-T3 augments erythropoiesis in vitro by stimulating local release of paracrine

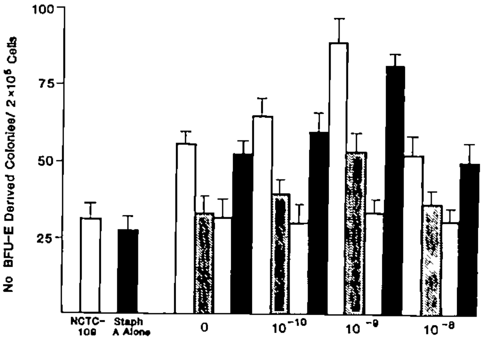

Fig 8. Removal of erythroid growth factors from CM supernatants. CM incubated for 24 hours with circulating leukocytes that were pulse exposed to L-T3 for 90 minutes or incubated without L-T3 (zero) were freed of membrane vesicles by high-speed centrifugation. Ten percent (vol/vol) of unabsorbed supernatants (open bars) and supernatants that were incubated sequentially with 100 µg (hatched bars) or 200 µg (stippled bars) of antimembrane IgG and then staphylococcal protein A were added to methylcellulose cultures. Control plates with 10% (vol/vol) NCTC-109, staphylococcal protein A alone, or normal rabbit IgG (200 µg) plus staphylococcal protein A (solid bars) were similarly tested. Mean ± SEM of bursts are shown for cultures containing 2.0 IU/mL human erythropoietin.

Table 2. Effects of L-T3 on CFU-GM Proliferation

Addition to Culture Underlayer	No Colonies/10^5 Cells
Experiment A	
NCTC-109	154 ± 14
CM	259 ± 28
CM + L-T3 (10^{-9} mol/L)	265 ± 23
L-T3 (10^{-9} mol/L)—direct addition	145 ± 20
Experiment B	
NCTC-109	127 ± 18
CM	180 ± 23
CM + L-T3 (10^{-10} mol/L)	169 ± 18
CM + L-T3 (10^{-9} mol/L)	185 ± 13
CM + L-T3 (10^{-8} mol/L)	178 ± 20
CM + L-T3 (10^{-7} mol/L)	189 ± 25
CM + L-T3 (10^{-6} mol/L)	195 ± 19
L-T3 (10^{-10} mol/L)—direct addition	115 ± 15
L-T3 (10^{-9} mol/L)—direct addition	123 ± 11
L-T3 (10^{-8} mol/L)—direct addition	139 ± 9
L-T3 (10^{-7} mol/L)—direct addition	125 ± 12
L-T3 (10^{-6} mol/L)—direct addition	140 ± 16

Mean ± SEM of colonies (containing ≥50 cells/clone) plus clusters (containing 3 to 49 cells/clone) are displayed for triplicate determinations hormones that, in turn, enhance BFU-E proliferation. First, bone marrow light-density mononuclear cells that are depleted of B lymphocytes by indirect panning with a monoclonal antibody that recognizes most normal B cells in peripheral blood and lymphoid organs[11] or by adherence to nylon wool[39,40] no longer respond to the mitogenic activity of L-T3 (see Table I). Sensitivity to L-T3 is restored by adding back the depleted cells. On the other hand, T cell lysis has no apparent effect on the ability of L-T3 to augment marrow burst formation. This might be expected since several investigators have shown that unlike their effects on circulating BFU-E proliferation, T lymphocytes appear to have a limited role in modulating the growth of normal human bone marrow BFU-E progenitors.[41–43] It has been suggested recently that B lymphocytes may be an important source of BPA or BPA-like factors that modulate early bone marrow erythroid differentiation processes.[44,45] Second, preincubation of BPA-producing peripheral blood and bone marrow cells with L-T3 results in biphasic dose-dependent augmentation of growth factor release that is similar to enhancement observed when L-T3 is added directly to culture (compare dose-response relationships in Fig 1 and 3). It is well known that hormones may show biphasic dose responses wherein growth-promoting effects are absent in vitro at a high-hormone concentration.[46] And third, L-T3 effects were found to be most pronounced at high marrow-seeding densities (Fig 2), suggesting that cellular interactions are important not only for burst formation to take place in vitro but also for the expression of erythropoietic activity by L-T3. Together, the data suggest that L-T3 augments the release of a factor(s) from B lymphocytes or monocytes that stimulates the proliferation of bone marrow BFU-E progenitors. Such a mitogen may act directly on the BFU-E, analogous to the action of T cell– and monocyte-derived BPA on peripheral blood erythroid progenitor cells.[47]

To determine the relatedness of L-T3–inducible proliferation factor(s) and BPA, we made kinetic measurements of the appearance of each during incubation. Both were detectable in CM within four hours from the onset of incubation with the hormone, a finding that is consistent with the release of other mammalian cell growth regulators and lymphokines following short incubation periods.[48–51] The rapidity of this effect may be explained by activity at a posttranscriptional level of protein synthesis. The rate of L-T3–induced factor release peaked earlier (three hours *v* 24 hours) and declined to a level below that of assayable BPA release by 24 hours, suggesting that L-T3 transiently augments early synthesis and/or exocytosis of lymphocyte mitogens. Peak rates of L-T3–induced protein release immediately preceded peak BPA release (compare data in Fig 5 and 7B). Successful absorption of L-T3–induced mitogens from CM supernatants with antiserum known to bind BPA[20] (Fig 8) is consistent with the hypothesis that inducible burst proliferation factors are antigenically similar to BPA.

A variety of human B lymphocyte plasma membrane components are known to be shed spontaneously into liquid culture medium.[52] We have previously shown that BPA is associated with plasma membrane–derived vesicles present in mitogen-free, liquid culture medium that has been incubated with lymphocytes.[10] Studies with antimembrane antiserum suggest that vesicle-associated BPA and BPA expressed by vesicle-free CM supernatant may share antigenic determinants.[20] To assess whether L-T3 augments the release of vesicle-associated mitogens, activity was assayed in component supernatant and pellet fractions of media conditioned by cells preincubated with L-T3. The results indicate that L-T3 preferentially enhances the release or expression of soluble BPA-like molecules. Furthermore, L-T3 effects can be overwhelmed by adding a saturating concentration of CM to the culture. This suggests that L-T3–induced molecules compete with and/or are functionally similar to BPA. Additional studies employing purified BPA and monoclonal antibodies directed against BPA[53] will be useful in examining this possibility and in probing the relationship of BPA expressed on membrane vesicles to that present in vesicle-free CM supernatants

Our results are distinct from those obtained with a transformed line of human erythroleukemia (K562) cells that form small colonies of embryonic hemoglobin-containing cells after short-term incubation.[54] Using this assay, Gauwerky and Golde found that 10^{-7} mol/L L-T3 induced colony formation in the complete absence of erythropoietin and other differentiation factors. Differences in results are not surprising since normal human bone marrow and transformed leukemic progenitors are known to respond to different chemical inducers (ie, Na butyrate, hemin, etc) and to have different erythropoietin requirements.[55–57] In addition, the time course of events suggests that such transformed cells are farther along the erythroid differentiation pathway than are the progenitors assayed here. Furthermore, studies with K562 cells were carried out in the presence of approximately 1% serum while those reported here were performed with biochemically defined additives. However, it is known that nuclei of hamster spleen erythroid cells bind radiolabeled thyroid hormone.[58] It is possible that as thyroid hormone receptors appear, direct effects upon more mature nucleated erythroblasts are discerned. This hypothesis is supported by observations of Fuhr and Dunn that globin synthesis is augmented in mouse fetal liver erythroid cells, an effect that is reversible by incubation with erythropoietin.[59] Their data suggest that thyroid hormone acts upon an erythroid cell that is later than the target cell of erythropoietin. To determine whether thyroid hormone also acts directly with specific nuclear receptors on normal human erythroid progenitor cells to stimulate their proliferation, additional studies will require availability of large numbers of highly purified progenitors.

The results of one line of experimentation indicate that L-T3–inducible growth factors are specific for the erythroid lineage. L-T3–inducible growth factors have no apparent activity on granulocyte-macrophage differentiation and/or growth (see Table 2). Whether CFU-megakaryocyte proliferation is altered by thyroid hormones is unknown.

In summary, our data suggest that rather than acting directly on BFU-E progenitors, L-T3 augments normal human bone marrow erythroid burst formation by enhancing the release of paracrine hormones from accessory leukocytes Other polypeptide hormones whose target tissues are widespread may express erythropoietic activity by a similar mechanism. Our results emphasize the importance of cellular interactions in regulating normal human erythropoiesis in vitro and possibly in vivo as well. They also point out the complex nature of tissue culture systems that are in widespread use today.

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REFERENCES

1. Golde DW, Bersch N, Cline MJ: Hormonal effects on erythroid stem cells, in Stamatoyannopoulos G, Nienhuis AW (eds). Cellular and Molecular Regulation of Hemoglobin Switching Orlando, FL, Grune & Stratton, 1978, p 305

2. Adamson JW: Hormonal interactions in erythropoiesis, in Stamatoyannopoulos G, Nienhuis AW (eds): Hemoglobins in Development and Differentiation. New York, Liss, 1981, p 73

3 Dainiak N: Role of defined and undefined serum additives to hematopoietic stem cell culture, in Cronkite EP, Dainiak N, McCaffrey RP, Palek J, Quesenberry PJ (eds): Hematopoietic Stem Cell Physiology. New York, Liss, 1985, p 59

4. Dainiak N, Hoffman R, Maffei LA, Forget BG: Potentiation of human erythropoiesis in vitro by thyroid hormone Nature 272:260, 1978

5 Iscove NN, Guilbert LJ Erythropoietin independence of early erythropoiesis and a two-regulator model of proliferative control in the hemopoietic system, in Murphy MJ (ed): In Vitro Aspects of Erythropoiesis New York, Springer-Verlag, 1978, p 3

6. Iscove NN: Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin-stimulated mouse spleen cells, in Golde DW, Cline MJ, Metcalf D, Fox CF (eds). Hemopoietic Cell Differentiation, ICN-UCLA Symposium on Molecular and Cellular Biology, X. Orlando, FL, Academic, 1978, p 37

7 Aye MT Erythroid colony formation in cultures of human marrow: Effect of leukocyte conditioned medium. Blood 44.69, 1977

8 Porter PN, Ogawa M, Leary AG: Enhancement of the growth of human early erythroid progenitors by bone marrow conditioned media Exp Hematol 8.83, 1983

9. Porter PN, Ogawa M: Characterization of human erythroid burst-promoting activity derived from bone marrow conditioned media. Blood 59.1207, 1982

10. Dainiak N, Cohen CM: Surface membrane vesicles from mononuclear cells stimulate erythroid stem cells to proliferate in culture. Blood 60:583, 1982

11. Nadler LM, Anderson KC, Marti G, Bates M, Park E, Daley JF, Schlossman SF: B4, a human B-lymphocyte–associated antigen expressed on normal, mitogen-activated and malignant B lymphocytes. J Immunol 131:244, 1983

12. Molinaro GA, Sato K, Paque RE, Dray S Rosetting of antibody-sensitized tumor cells with anti-immunoglobulin antibody-coated erythrocytes by a new method for detecting antigens on cells. Cancer Res 35:2213, 1975

13 Indiveri F, Wilson BS, Pelligrino MA, Ferrone S: Detection of human histocompatibility (HLA) antigens with an indirect rosette microassay. J Immunol Methods 29:101, 1979

14 Dainiak N, Kreczko S, Cohen AM, Pannell R, Lawler J: Primary human marrow cultures for erythroid bursts in a serum-substituted system. Exp Hematol 13:1073, 1985

15 Dainiak N, Davies G, Kalmanti M, Lawler J, Kulkarni V: Platelet-derived growth factor promotes proliferation of erythropoietic progenitor cells in vitro J Clin Invest 71:1206, 1983

16. Dainiak N, Kreczko S Interactions of insulin, insulinlike growth factor II, and platelet-derived growth factor in erythropoietic culture. J Clin Invest 76:1237, 1985

17 Stroobant P, Waterfield MD: Purification and properties of porcine platelet–derived growth factor. EMBO J 12:2963, 1984

18. Marquardt H, Todaro GJ, Henderson LE, Oroszlan S: Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures J Biol Chem 256:6859, 1981

19. Pike BL, Robinson WA: Human bone marrow colony growth in agar gel J Cell Physiol 76:77, 1970

20. Dainiak N, Feldman L, Cohen CM. Neutralization of erythroid burst-promoting activity in vitro with antimembrane antibodies Blood 65:877, 1985

21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193.265, 1951

22. Laemmli VK Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227:680, 1970

23. Dzandu JK, Deh ME, Barratt DL, Wise GE: Detection of erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same polyacrylamide gel using a double-staining technique. Proc Natl Acad Sci USA 81.1733, 1984

24 Goldstein A: Biostatistics, an Introductory Text. New York, Macmillan, 1964, p 59

25. Cline MJ, Golde DW: Production of colony-stimulating activity by human lymphocytes. Nature 248 703, 1974

26. Parker JW, Metcalf D: Production of colony-stimulating factor in mitogen-stimulated lymphocyte cultures. J Immunol 112:502, 1974

27. Ruscetti FW, Chervenick PA. Regulation of the release of colony-stimulating activity from mitogen-stimulated lymphocytes J Immunol 114:1513, 1975

28. Gibson JG, Harris AW: Clinical studies of the blood volume. V. Hyperthyroidism and myxedema. J Clin Invest 18 59, 1939

29. Muldowney FP, Crooks J, Wayne EJ: The total red cell mass in thyrotoxicosis and myxoedema. Clin Sci 16:309, 1957

30. Fein HG, Rivlin RS Anemia in thyroid diseases. Med Clin North Am 59:1133, 1975

31. Horton L, Coburn RJ, England JM, Himsworth RL: The haematology of hypothyroidism Quart J Med 45.101, 1976

32. Bomford R: Anaemia in myxoedema: And the role of the thyroid gland in erythropoiesis Q J Med 7:495, 1938

33. Donati RM, Fletcher JW, Wamecke MA, Gallagher, NI: Erythropoiesis in hypothyroidism. Proc Soc Exp Biol Med 144:78, 1973

34. Peschle C, Zanjani ED, Gidari AS, McLaurin ND, Gordon AS Mechanisms of thyroxine action on erythropoiesis. Endocrinology 89:609, 1971

35. Das KC, Mukherjec M, Sarkar TK, Dash RJ, Rastogi GK: Erythropoiesis and erythropoietin in hypo and hyperthyroidism J Clin Endocrinol Metab 40:211, 1975

36. Popovic WJ, Brown JE, Adamson JW: The influence of thyroid hormones on in vitro erythropoiesis. Mediation by a receptor with beta-adrenergic properties. J Clin Invest 60:907, 1977

37. Golde DW, Bersch N, Chopra IJ, Cline MJ: Thyroid hormones stimulate erythropoiesis in vitro. Br J Haematol 37.173, 1977

38. Fuhr JE, Dunn CDR: Thyroid hormones and protein synthesis in fetal mouse liver erythroid cells. Exp Hematol 7.490, 1979

39. Eisen JA, Wedner HJ, Parker CW: Isolation of pure human peripheral blood T-lymphocytes using nylon wool columns. Immunol Commun 1:571, 1972

40. Greaves MF, Brown G: Purification of human T and B lymphocytes. J Immunol 112:420, 1974

41. Zuckerman KS· Human erythroid burst forming units Growth in vitro is dependent on monocytes but not T-lymphocytes. J Clin Invest 67·702, 1981

42. Lipton JM, Reinherz EL, Kudisch M, Jackson PL, Schlossman SF, Nathan DG: Mature bone marrow erythroid burst-forming units do not require T cells for induction of erythropoietin-dependent differentiation. J Exp Med 152:350, 1980

43. Kirshner J, Ozer H, O'Leary M, Higby D, Marinello M, Preisler H: T-cell depletion of bone marrow does not inhibit in vitro hematopoiesis. Blut 50:201, 1985

44. Najman A, Baillou C, Drouet X, Leblanc G, Donay L, Gorin NC, Duhamel G: Regulation of human peripheral blood BFU-E growth in vitro in leukaemic B-lymphocytes. Br J Haematol 60:643, 1985

45. Dainiak N, Najman A, Kulkarni V, Sutter D, Kreczko S, Feldman L, Baillou C, Leblanc G: Regulation of erythroid burst proliferation by B-lymphocytes from healthy donors and patients with chronic lymphocytic leukemia (CLL). Blood 64:113, 1984 (suppl 1)

46. Barnes D, Sato G: Methods for growth of cultured cells in serum-free medium. Anal Biochem 102:255, 1980

47. Linch DC, Nathan DG: T cell and monocyte-derived burst-promoting activity directly act on erythroid progenitor cells. Nature 312.775, 1984

48. Hall DJ, O'Leary JJ, Rosenberg A: Early synthesis of specific cytoplasm proteins is correlated with the rate of exit of lymphocytes from the resting state. J Cell Biol 99:1814, 1984

49. Barth E-M, Korsching S, Thoenen H: Regulation of nerve growth factor synthesis and release in organ cultures of rat iris. J Cell Biol 99:839, 1984

50. Szabo MT, Hrabak A, Antoni F: An inhibitory factor of DNA synthesis derived from the medium of short-time incubated human lymphocytes. Immunol Lett 1:115, 1979

51 Prystowsky MB, Ely JM, Beller DI, Eisenberg L, Goldman J, Goldman M, Goldwasser E, Ihle J, Quintans J, Remold H, Vogel SN, Fitch FW: Alloreactive cloned T cell lines VI Multiple lymphokine activities secreted by helper and cytolytic cloned T lymphocytes. J Immunol 129:2337, 1982

52. Caraux J, Serrou B: Release of Fc-binding component from normal or activated human lymphocytes. Ann NY Acad Sci 77:101, 1979

53. Dainiak N, Sutter D: Suppression of human marrow erythroid colony formation (ECF) by hybridoma antibodies raised against membrane-derived vesicles shed by lymphocytes into conditioned medium (CM). Clin Res 33:337, 1985

54 Gauwerky C, Golde D. Hormonal effects on cell proliferation in a human erythroleukemia cell line (K562). Blood 56:886, 1980

55 Hoffman R, Murnane MJ, Benz EJ, Prohaska R, Floyd V, Dainiak N, Forget BJ, Furthmayr H: Induction of erythropoietic colonies in a human chronic myelogenous leukemia cell line. Blood 54·1182, 1979

56 Rutherford TR, Clegg JB, Weatherall DJ: K562 human leukaemic cells synthesize embryonic haemoglobin in response to haemin. Nature 280:164, 1979

57. Benz EJ, Murnane MJ, Tonkonow BL, Berman BW, Mazur EM, Cavallesco C, Jenko T, Snyder EL, Forget BG, Hoffman R. Embryonic-fetal erythroid characteristics of a human leukemic cell line. Proc Natl Acad Sci USA 77:3509, 1980

58. Boussios T, McIntyre WR, Gordon AS, Bertles JF: Receptors specific for thyroid hormones in nuclei of mammalian erythroid cells: Involvement in erythroid cell proliferation. Br J Haematol 51:99, 1982

59. Fuhr JE, Dunn DR: Thyroid hormones and protein synthesis in fetal mouse liver erythroid cells. Exp Hematol 7.490, 1979

Denaturant-Induced Stimulation of the β-Migrating Plasminogen Activator Inhibitor in Endothelial Cells and Serum

By Larry A. Erickson, Carla M. Hekman, and David J. Loskutoff

Cultured bovine aortic endothelial cells and human serum contain plasminogen activator inhibitors (PAIs) that are immunologically related. In the present study, the electrophoretic mobilities, molecular weights (mol wt), and activities of these PAIs were compared. When fractionated by agarose zone electrophoresis, both PAIs migrated with β mobility as compared with the mobilities of human plasma/serum proteins. Two-dimensional electrophoretic analysis, employing agarose zone electrophoresis in the first dimension and sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the second dimension, indicated that these β-PAIs comigrated, both having a mol wt of approximately 50,000. The activity of the PAI in endothelial cell–conditioned medium is enhanced severalfold by treatment with either sodium dodecyl sulfate or guanidine. In preliminary experiments, we were unable to stimulate the PAI activity of undiluted serum by similar treatments. However, the PAI activities in both diluted serum and gel-filtered or electrophoretically fractionated serum were enhanced by treatment with these denaturants. The gel filtration studies also revealed that serum contains multiple forms of the β-PAI. These forms may represent polymeric PAI and/or complexes between the PAI and other serum components. These findings indicate that the primary PAIs in bovine endothelial cells and human serum are not only immunologically related but are also biochemically similar.

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THE GENERATION of plasmin from plasminogen provides an important source of proteolytic activity in tissues and biologic fluids.[1,2] Precise regulation of plasminogen activator (PA) activity thus may constitute a critical feature of many biologic systems.[3] For example, in the vascular system, the abnormal expression of PA is associated with the development of both thrombotic and bleeding disorders.[4,5] The factors that regulate vascular PA activity are not entirely understood. Until recently, such control was thought to be at the level of the formation and resolution of fibrin itself[2,6] rather than through the action of specific PA inhibitors (PAIs).[7] However, the recent demonstration that PAIs not only exist in plasma[8–10] but are frequently detected in complex with tissue-type PA (tPA)[11,12] suggests that vascular PA activity is also regulated by these inhibitors. The observations that a PAI is also present in platelets[13,14] and can be released from them by physiological stimuli[14] and that the level of PAI in plasma may be altered in certain human diseases[15,16] support this hypothesis.

Available evidence indicates that there are at least three immunologically distinct PAIs, including the endothelial cell–type inhibitor,[17] the placental inhibitor,[18] and protease nexin.[19] The PAI synthesized by cultured bovine aortic endothelial cells (BAEs) has been purified and partially characterized.[17] It inhibits both urokinase and tPA, but not plasmin. Antiserum to it has been developed and employed to demonstrate that the endothelial cell, plasma, serum, and platelet PAIs are immunologically related.[20] This class of PAIs has a number of unusual properties that distinguish it from other cellular and blood-derived protease inhibitors. For example, it is stable under conditions that inactivate other inhibitors (eg, incubation at pH 3 or exposure to sodium dodecyl sulfate [SDS]).[17,21] Moreover, the activity of the PAI from BAEs is stimulated by treatment with denaturants such as guanidine and SDS.[22] The objective of the current study was to compare further the endothelial cell and serum PAIs. In this report, we demonstrate that both of these inhibitors have β mobility as determined by agarose zone electrophoresis, and the activity of both can be stimulated by denaturants.

MATERIALS AND METHODS

Cell culture. BAEs were isolated and maintained in vitro in modified minimum essential medium (MEM; Flow Laboratories, Inglewood, CA) containing 10% calf serum (Irvine Scientific, Santa Ana, CA or Sterile Systems, Inc, Logan, UT) as described[23] The cells used for these studies were cloned from a single cell[17,24] and had been passaged five to 20 times. Conditioned medium (CM) was prepared by incubating washed cell monolayers in serum-free MEM for 24 hours at 37°C. This CM was collected, centrifuged at 1,000 g to remove floating cells and cellular debris, and stored in 0.01% Tween 80 (J.T. Baker Chemical Co, Phillipsburg, NJ) at −20 °C until used For some experiments, 200 μL of CM were concentrated tenfold by centrifugation in a SpeedVac concentrator (Savant, Farmingdale, NY).

Preparation of plasma and serum Human blood was collected from healthy volunteers into polypropylene tubes containing 3.8% Na citrate, pH 7 4, and centrifuged at 1,000 rpm for 15 minutes at 23 °C to obtain platelet-rich plasma. The platelet-rich plasma was chilled to 4 °C and centrifuged at 3,000 rpm for 30 minutes to obtain platelet-poor plasma (hereafter referred to as plasma) Whole blood serum was prepared by collecting blood into glass tubes, allowing the blood to clot at 37 °C for one hour, and then removing the clot by centrifugation at 3,000 rpm for 20 minutes at 4 °C. The resulting serum was collected and stored at −70 °C until used

SDS and guanidine treatment of samples. BAE CM and human whole blood serum, either undiluted or diluted tenfold with phos-

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phate-buffered saline (0.14 mol/L NaCl, 0.01 mol/L Na phosphate, pH 7.2, containing 0.01% Tween 80; PBS/Tw), were incubated with SDS (0.1% or 0.25% final concentration as indicated) for 45 minutes at 37 °C. Alternatively, the samples were dialyzed against 4 mol/L guanidine-HCl (in PBS/Tw adjusted to pH 7.2) for 4.5 hours at 37 °C. Dialysis was judged complete by the disappearance of the phenol red present in the CM. After treatment with SDS or guanidine, samples were dialyzed against 4,000 volumes of PBS/Tw at 4 °C for 18 hours.

Electrophoresis. SDS-polyacrylamide slab gels and buffers were prepared as described by Laemmli.[25] Samples were applied to gels consisting of 10-cm resolving gels of 9% acrylamide and 2-cm stacking gels of 4% acrylamide and then subjected to electrophoresis at room temperature for 16 hours or until the tracking dye reached the bottom of the gel.

Agarose zone electrophoresis was performed in 1% agarose gels using 0.025 mol/L Tris-tricine, pH 8.6 (Bio-Rad Laboratories, Richmond, CA), as the electrode buffer. Samples were subjected to electrophoresis at 12 mA/10-cm gel for three hours using a water-cooled horizontal electrophoresis apparatus (Bio-Rad). A sample of human plasma (4 μL) was used as a reference marker. Under the conditions employed, the albumin present in the plasma migrated in an anodal direction approximately 60 mm from the origin. The plasma proteins were stained with 0.2% Coomassie brilliant blue R250 (Bio-Rad) in 14% acetic acid and 28% isopropanol. In some experiments, lanes of the agarose gel were treated with 0.1% 2-mercaptoethanol (2-ME) and either 0.1% SDS or 4 mol/L guanidine-HCl at 37 °C for 30 minutes and then washed extensively in 2.5% Triton X-100 (Bio-Rad, Richmond, CA) or PBS prior to further analysis. These treatments inactivate cellular PAs (2-ME) or stimulate PAI activity (SDS, guanidine). In other experiments, the agarose gels were cut into 5-mm slices. The material that eluted from each slice upon extraction into 150 μL of PBS for four hours at 4 °C was then analyzed for PAI activity in the tPA-binding assay (see the following material).

Two-dimensional electrophoresis was performed by using a combination of the two electrophoretic techniques just outlined. Samples were first subjected to zone electrophoresis in an agarose gel. After electrophoresis, the agarose gel was soaked in electrophoresis sample buffer (0.125 mol/L Tris, pH 6.8, containing 2.2% SDS and 20% glycerol) for 30 minutes and placed on top of a 10-cm resolving 9% acrylamide slab gel, and then SDS-polyacrylamide gel electrophoresis (PAGE) was performed as just outlined. The agarose and polyacrylamide gels were analyzed by reverse fibrin autography (RFA), immunoblotting, or autoradiography, as will be described.

Sephacryl S-200 chromatography of human serum. Four and one-half milliliters of human whole blood serum were incubated with diisopropylfluorophosphate (DFP, 5 mmol/L final concentration; Sigma Chemical Co, St Louis) for three hours at 4 °C. The serum was then applied to a Sephacryl S-200 column (1.5 cm × 110 cm) that had been equilibrated in 10 mmol/L Tris-HCl, pH 7.6, 0.01% Tween 80, and 3 mol/L NaCl, and calibrated with mol wt standards (Sigma). The standards included blue dextran (mol wt, 2×10^6), bovine serum albumin (mol wt, 67,000), carbonic anhydrase (mol wt, 30,000), lysozyme (mol wt, 14,400), and phenol red (mol wt, 354). The column was eluted at 12 mL/h and 2.0-mL fractions were collected. Aliquots of the fractions were treated with either 0.25% SDS or 4 mol/L guanidine-HCl as described before, and both treated and untreated samples were dialyzed against PBS/Tw at 4 °C for 18 hours. These samples were then assayed for PAI activity in the tPA-binding assay.

Assays for PAI. PAI was quantified by using a variety of assays. For example, RFA was performed on samples subjected to agarose or SDS-polyacrylamide slab gel electrophoresis as outlined previously.[26] Immunoblotting of material transferred from agarose gels to nitrocellulose was performed as described.[27] The transferred proteins were first incubated with monospecific rabbit antiserum (diluted 1:500) against the BAE PAI,[17] and the bound rabbit IgG was then detected with goat antirabbit IgG (Cooper Biomedical, Inc, Malvern, PA) that had been ^{125}I labeled. The ^{125}I-labeled IgG was used at a final concentration of 200,000 cpm/mL. Between incubations with the antibodies, the nitrocellulose paper was rinsed extensively with PBS containing 5% (wt/vol) nonfat dry milk, 0.01% Antifoam A (Sigma), and 0.0001% sodium ethylmercurithiosalicylate (thimerosal).[28] It was then air-dried and subjected to autoradiography on Kodak X-omat film (Rochester, NY) for 24 hours at −70 °C.

PAI activity was also assessed by measuring the ability of components in CM and serum samples to form SDS-stable complexes with tPA. Two approaches were taken. In the first, the samples were incubated with ^{125}I-labeled tPA for ten minutes at 37 °C. The mixtures were then fractionated by SDS-PAGE, and the gels were fixed and stained in 0.01% Coomassie brilliant blue R250/50% trichloroacetic acid, destained in 10% acetic acid, dried, and subjected to autoradiography on Kodak X-omat film for 24 hours at −70 °C. High mol wt complexes were easily distinguished from free tPA by this approach. In the second method, the ability of the PAI to bind to tPA immobilized on microtiter wells was assessed in the tPA-binding assay, performed essentially as described by Schleef et al.[29] The amount of bound PAI was determined by using the antiserum against the BAE PAI.

Miscellaneous. Plasminogen was prepared by affinity chromatography on lysine-Sepharose as described.[30] Human α-thrombin was a gift from J. Fenton (New York State Department of Health, Albany). Fibrinogen (bovine, plasminogen- and thrombin-free) was purchased from Calbiochem-Behring (San Diego, CA), α_2-antiplasmin from American Diagnostica Inc (Greenwich, CT), 2-ME and SDS from Bio-Rad, guanidine-HCl from Sigma, and culture supplies from Falcon Labware (Oxnard, CA). Mol wt standards used for SDS-PAGE were obtained from Bio-Rad and included carbonic anhydrase (mol wt, 31,000), bovine serum albumin (mol wt, 66,200), and β-galactosidase (mol wt, 116,250). The tPA was purified according to previously described procedures.[31] Both the tPA and the goat antirabbit IgG were labeled with ^{125}I (Amersham Corp, Arlington Heights, IL) by the Iodogen method[32] modified such that the labeling time was 3.5 minutes at 4 °C. The specific activity of the ^{125}I-labeled tPA was approximately 7.1×10^7 cpm/μg of protein. Immunodepletion experiments were performed as described previously[17,20] using nonimmune or anti-PAI IgG coupled to protein A–coated Sepharose CL-4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden).

RESULTS

Analysis of BAE CM by zone and two-dimensional electrophoresis. Samples of BAE CM were subjected to agarose zone electrophoresis and then analyzed for PAI activity by RFA and for PAI antigen by immunoblotting (Fig 1). Although no PAI activity was detected in 50 μL of CM (panel B, lane 1), immunoblotting demonstrated that this same amount of CM did contain PAI antigen (panel B, lane 3). The antigen exhibited β mobility when compared with known plasma proteins (panel A). Inhibitor activity could be detected in the β region of the gel when 200 μL of CM was analyzed (panel B, lane 2). These results indicate that under the electrophoretic conditions employed, the PAI in BAE CM migrates with β mobility relative to the migration of plasma proteins.

We have shown previously that 5% or less of the total PAI in BAE CM is active, the other 95% existing in a latent, or

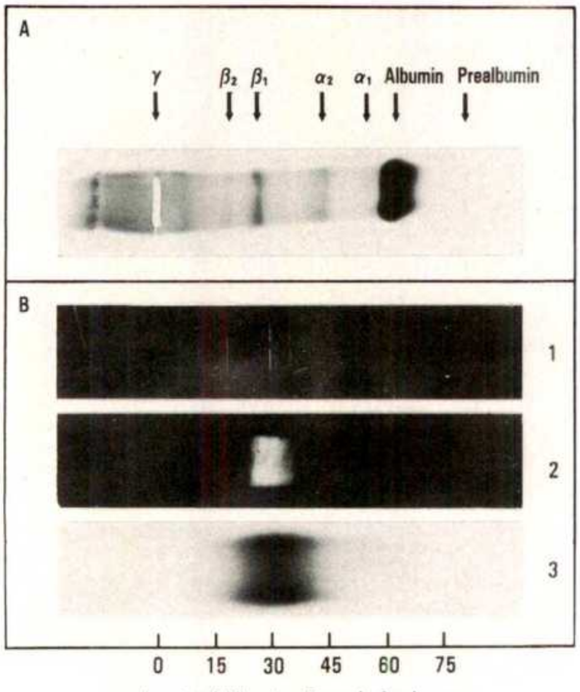

Fig 1. Zone electrophoresis of BAE CM. The mobility of the PAI in BAE CM was compared with the mobilities of proteins in human plasma. Human plasma (4 μL, panel A) and BAE CM (panel B) were subjected to agarose zone electrophoresis as described in Materials and Methods. The plasma proteins were stained with 2.5% Coomassie brilliant blue R250, and the CM samples were analyzed by RFA (lanes 1 and 2) or by immunoblotting (lane 3) using antiserum against purified BAE PAI. In panel B, either 50 μL of CM (lanes 1 and 3) or 20 μL of tenfold concentrated CM (lane 2) were analyzed.

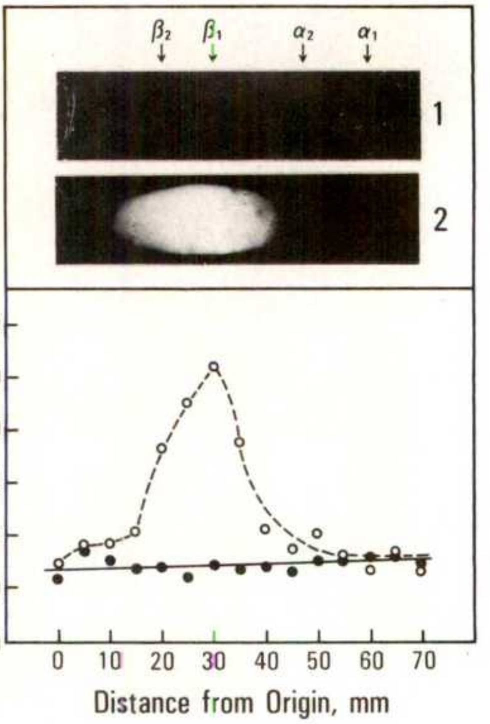

Fig 2. SDS activation of the β-PAI in BAE CM. Four aliquots of BAE CM (50 μL each) were subjected to zone electrophoresis. Two of the gel lanes were treated with 0.1% SDS containing 0.1% 2-ME as described in Materials and Methods, and the other two gels served as untreated controls. One untreated gel (lane 1, top panel) and one SDS-treated gel (lane 2, top panel) were analyzed by RFA. The other two gels were sliced into 5-mm sections. Each section was then extracted into buffer, and the resulting extracts (50 μL) were analyzed for PAI activity in the tPA-binding assay (bottom panel). Untreated, ●; SDS/2-ME–treated, ○. The relative mobilities of plasma proteins are indicated by the arrows at the top and were determined as described in the legend for Fig 1.

inactive, form.[22] The latent form can be converted into the active form by treatment with denaturants. The effect of SDS on the PAI activity of BAE CM fractionated by agarose zone electrophoresis is shown in Fig 2. Again, no PAI activity was detected in any region of the gel when 50 μL of untreated CM was analyzed (Fig 2, top panel, lane 1). However, when the agarose gel containing the fractionated CM was soaked in SDS containing 2-ME, washed to remove these reagents, and then analyzed by RFA, a prominent PAI zone was detected in the β region (lane 2). This stimulation of PAI activity by SDS was quantified by using the tPA-binding assay (Fig 2, bottom panel). In this case, the SDS-treated and untreated gels were sliced, and the material in each slice was eluted into buffer and then assessed for PAI activity. Again, no PAI activity was detected in the untreated fractions, whereas pronounced PAI activity was detected in fractions obtained from the β region of the SDS-treated gel. β-PAI activity (Fig 3, lane 1) was not detected in SDS-treated agarose gels containing CM previously incubated with anti-PAI serum bound to protein A–Sepharose beads (lane 2); however, it was apparent in SDS-treated agarose gels containing CM pretreated with nonimmune serum (lane 3). Thus, the β-PAI activity revealed by SDS treatment of CM after agarose zone electrophoresis is immunologically related to the 50,000–mol wt PAI purified from BAEs.

The electrophoretic properties of the PAI and α_2-antiplasmin, the primary plasmin inhibitor in blood,[2,33] were compared (Fig 4). Purified human α_2-antiplasmin was added to BAE CM, and the mixture was fractionated by agarose zone electrophoresis, by SDS-PAGE, or by two-dimensional gel electrophoresis. As expected, no inhibitor activity was apparent in the β region of the agarose gel (panel A), in agreement with the results shown in Figs 1 and 2. However, a very prominent inhibitor zone was seen in the α_2 region, presumably corresponding to α_2-antiplasmin. This relationship changed dramatically when the inhibitors in the agarose gel were subsequently subjected to electrophoresis into a SDS-containing gel (panel B). Considerable inhibitor activity was now apparent in the β region, whereas the inhibitor activity in the α_2 region was greatly decreased. These changes reflect the different effects of SDS on the biologic activities of the two inhibitors. Panel C shows the mixture fractionated by SDS-PAGE alone. Inhibitor activity was detected at a mol wt of 70,000 (the mol wt of α_2-antiplasmin) and at a mol wt of 50,000 (the mol wt of the PAI). The proteins responsible for these activities comigrated with those shown in panel B.

Effect of SDS and guanidine on the PAI activity of serum. We have shown previously that the primary PAI in human serum is immunologically related to the BAE PAI.[20] Experiments were performed to determine whether the activ-

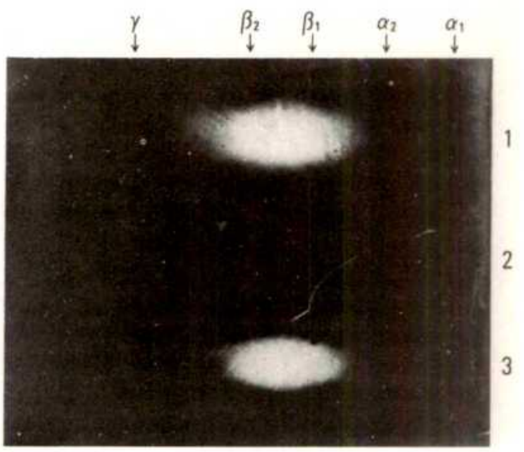

Fig 3. Immunoadsorption of the β-PAI in BAE CM. BAE CM (50 μL, lane 1) was immunoprecipitated with anti-PAI serum (lane 2) or nonimmune serum (lane 3) as described in Materials and Methods. Each of the resulting supernatant fluids was subjected to agarose zone electrophoresis. The agarose gels were then treated with 0.1% SDS and 0.1% 2-ME as described in the legend to Fig 2 and assayed for inhibitor activity by using RFA. The mobilities of plasma proteins are indicated.

ity of the serum PAI, like that of the BAE PAI, could be stimulated by SDS and/or guanidine. In these experiments, PAI activity was assessed by first adding ^{125}I-labeled tPA to the sample and then visualizing the amount of tPA-PAI complex formed by using SDS-PAGE and autoradiography (Fig 5). ^{125}I-labeled tPA alone is shown in lane 1. The amount of complex formed using BAE CM as the source of PAI increased upon SDS-treatment (lanes 2 and 3), in agreement with our previously reported results with BAE CM.[22] However, there was no SDS-mediated increase in the amount of complex formed when serum was employed as the PAI source (compare lanes 4 and 5). Unexpectedly, when these same serum samples were analyzed by RFA, considerable 50,000–mol wt PAI activity was detected (data not shown). Thus, undiluted whole blood serum appeared to contain PAI activity that could be detected after SDS-PAGE and RFA, but could not be revealed by direct treatment with SDS. Experiments were performed to test the possibility that the large amount of protein present in the serum bound most of the added SDS and thus prevented it from interacting with the PAI and enhancing its activity. In these experiments, we examined the effect of SDS or guanidine on the PAI activity in either fractionated or diluted serum.

Serum was subjected to agarose zone electrophoresis, and the gels were treated with either 0.1% SDS, 4 mol/L guanidine-HCl (both containing 0.1% 2-ME to inactivate PAs), or buffer alone. The gels were then washed with buffer (containing 2.5% Triton X-100) and assessed for inhibitor activity by RFA (Fig 6). A single zone of inhibition with α_2 mobility (presumably α_2-macroglobulin and/or α_2-antiplasmin) was apparent in the untreated gel (lane 1) but was absent in both the SDS- and guanidine-treated gels (lanes 2 and 3). However, both of the treated gels exhibited a zone of inhibition in the β region (lanes 2 and 3), which was not at all evident in the untreated gel (lane 1). The latent PAI in serum was also apparent after two-dimensional gel electrophoresis (Fig 7). Thus, when serum previously fractionated by agarose gel electrophoresis (top panel) was subsequently subjected to SDS-PAGE (bottom panel), a new PAI was revealed. It migrated with β mobility and had a mol wt of approximately 50,000. In these experiments, serum α_2-antiplasmin activity was detected after both zone electrophoresis (Fig 7, top panel) and two-dimensional electrophoresis (Fig 7, bottom panel); however, most of it was inactivated by the second-dimension SDS-PAGE, in agreement with the results shown in Fig 4. No β-PAI activity was detected in normal human plasma although it was present in plasma that contained elevated fast-acting PAI activity[8-10] and in platelets (data not shown). In all cases in which the 50,000–mol wt β-PAI was detected after two-dimensional electrophore-

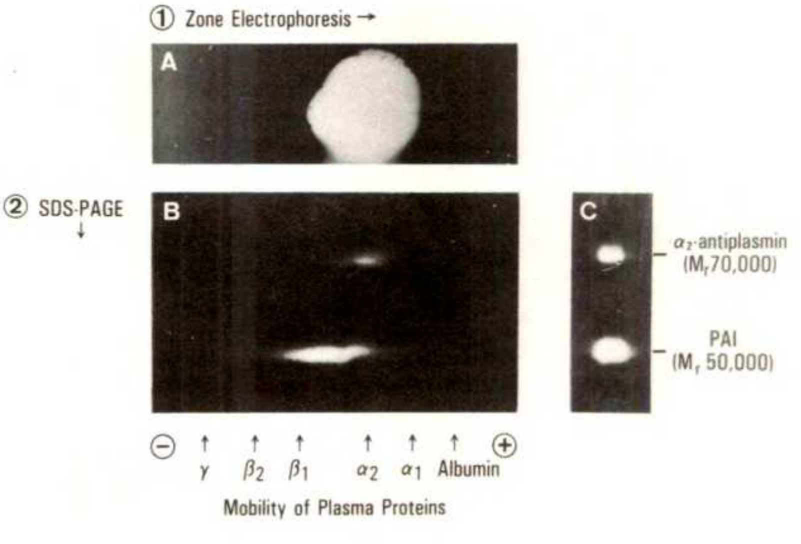

Fig 4. Analysis of BAE CM and α_2-antiplasmin by two-dimensional electrophoresis. BAE CM (15 μL) was combined with α_2-antiplasmin (25 μL, 500 ng) and then subjected to either agarose zone electrophoresis (panel A), agarose zone electrophoresis followed by SDS-PAGE (panel B), or SDS-PAGE alone (panel C). Each gel was then analyzed for inhibitors by using RFA. The electrophoretic mobilities of the inhibitors fractionated by agarose zone electrophoresis (panel A) were compared with those of proteins in human plasma as described in the legend to Fig 1 (arrows, bottom).

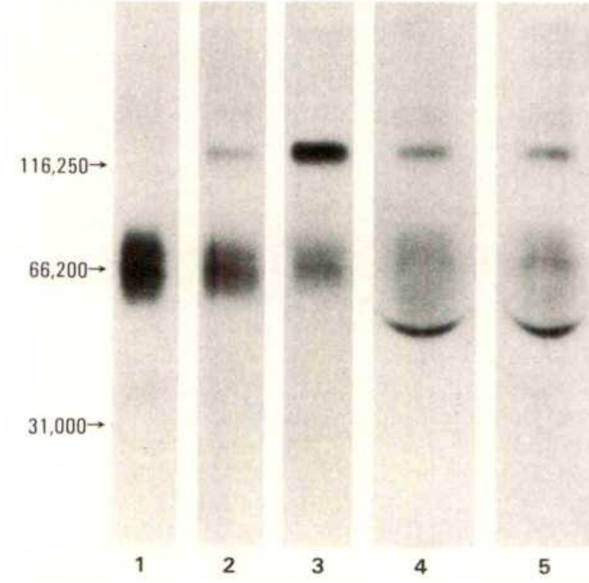

Fig 5. Formation of complexes between ^{125}I-labeled tPA and the PAIs in BAE CM and human serum. ^{125}I-labeled tPA (approximately 0.3 ng, 26,000 cpm, lane 1) was incubated for ten minutes at room temperature with untreated BAE CM (92 μL, lane 2), with untreated human serum (10 μL, lane 4), with SDS-treated BAE CM (92 μL, lane 3), or with SDS-treated human serum (10 μL, lane 5). The samples were then subjected to SDS-PAGE and autoradiography. The positions of the mol wt standards are indicated. The mobility of the ^{125}I-labeled tPA remaining free in the serum (lanes 4 and 5) was distorted by the large amount of albumin present (compare with lane 1; see refs 14 and 20).

sis, it was also recognized by antiserum against the PAI purified from BAE CM (data not shown).

Whole blood serum was also fractionated by Sephacryl S-200 chromatography as described in Materials and Methods. Aliquots of the fractions were then treated with 0.25% SDS or 4 mol/L guanidine-HCl, dialyzed into PBS/Tw, and assayed for PAI activity in the tPA-binding assay (Fig 8). The only tPA-binding activity detected in untreated control fractions was in the material that eluted just after the void volume of the column (apparent mol wt between 150,000 and 200,000). Treatment of the fractions with either SDS or guanidine-HCl reduced this activity but induced the appearance of two additional peaks of PAI activity. The first of these migrated with an apparent mol wt of 90,000 and was stimulated equally well by both SDS and guanidine-HCl. The second peak migrated with an apparent mol wt of 30,000 and was stimulated to a greater extent by guanidine-HCl than by SDS. This latter result is consistent with our previous findings that the latent PAI in BAE CM is activated to a greater extent by guanidine-HCl than by SDS and that it migrates with an apparent mol wt of 30,000 when fractionated by Sephacryl S-200 chromatography prior to treatment with denaturant.[22]

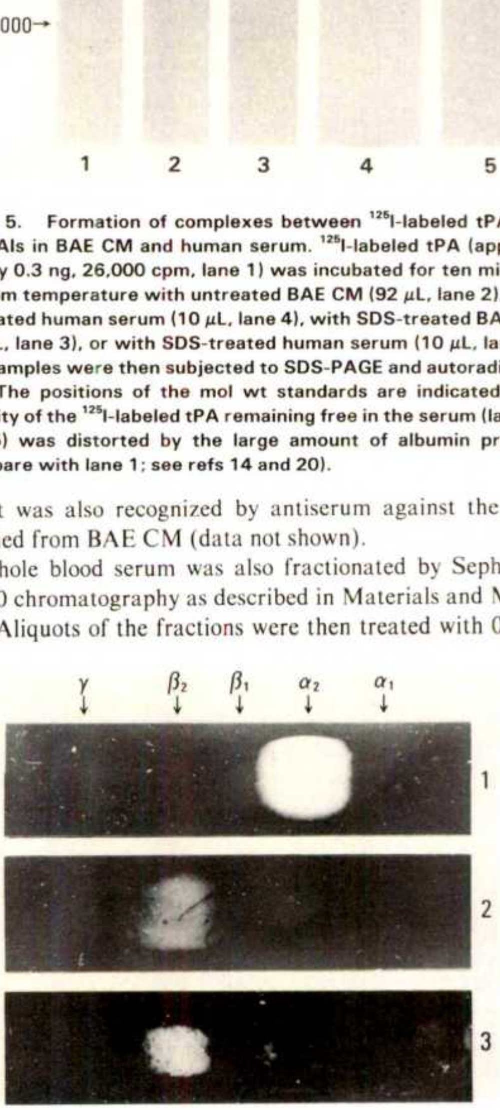

Fig 6. Activation of the serum β-PAI after zone electrophoresis. Triplicate samples of human serum (25 μL) were subjected to zone electrophoresis. One lane of the agarose gel was untreated (lane 1) and the other lanes were treated with either 0.1% SDS (lane 2) or with 4 mol/L guanidine-HC1 (lane 3), both in the presence of 0.1% 2-ME as described in Materials and Methods. All of the gel lanes were then analyzed for inhibitors by RFA. The relative mobilities of serum proteins are indicated by the arrows at the top and were determined as described in the legend to Fig 1.

To determine whether activation of the serum PAI requires the prior removal of other serum proteins or can be achieved by merely diluting the sample, BAE CM and human serum (either undiluted or diluted tenfold) were treated with 0.25% SDS or dH_2O as described in Materials and Methods. All samples were than dialyzed into PBS/Tw, brought to the same final dilution, and assayed for PAI activity in the tPA-binding assay. Table 1 shows that dilution of BAE CM prior to treatment with denaturant had no effect on the enhancement of PAI activity. Untreated CM exhibited between 4 and 8 ng/mL of active PAI, whereas both undiluted SDS-treated CM and CM treated after dilution exhibited approximately 500 ng/mL of PAI activity. In contrast, when undiluted serum was SDS-treated, the amount of detectable PAI actually decreased when compared with untreated serum. However, when the serum was diluted before SDS treatment, the amount of PAI activity detected increased by a factor of three (sample 1) and 27 (sample 2).

DISCUSSION

PAs, in addition to their role in initiating vascular fibrinolysis, have been implicated in a variety of other physiological processes including ovulation, cell migration, embryo implantation, epithelial differentiation, malignant transformation, and activation of latent collagenase.[3] PAI activities have now been detected in many of the cells and fluids associated with these processes,[34-37] thus emphasizing their potential role in regulating PA-mediated events. This PAI activity may result from the presence of either the endothelial cell–type PAI, the placental-type PAI, or from protease nexin. Unfortunately, most available assays cannot readily distinguish between these PAIs. This ambiguity is further emphasized by the finding that each of these PAIs appears to be widely distributed, and in some cases, one type of cell may produce more than one PAI. In fact, the human fibrosarcoma cell line, HT-1080, appears to produce all three PAIs.[38,39] It is also clear that cells that do not produce one of the PAIs under one set of conditions may be induced to do so under another set of conditions.[34,35,39,40] These considerations emphasize not only the ambiguity of PAI activity measure-

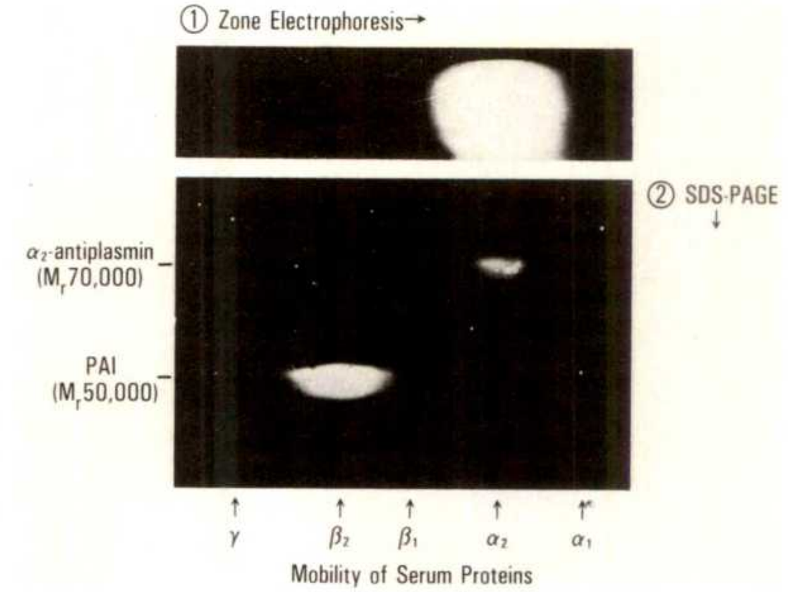

Fig 7. Analysis of human serum by two-dimensional electrophoresis. Human serum (25 μL) was subjected to zone electrophoresis (top panel) or to two-dimensional electrophoresis (bottom panel) as described in Materials and Methods. Both gels were then analyzed for inhibitors by RFA. The mobilities of the detected inhibitors were compared to those of proteins in human serum (arrows) as described in the legend to Fig 1.

ments but also the shortcomings associated with the use of the cell or tissue first identified with a particular PAI as the basis for developing a nomenclature for these molecules.

The results presented here identify two properties that may be unique to the endothelial-type PAIs and that may aid in the development of a meaningful PAI nomenclature. First, we have demonstrated that the PAI in BAE CM (Figs 1 to 4) as well as the immunologically related PAI in human serum (Figs 6 and 7) have β mobility as determined by agarose zone electrophoresis. The primary PAIs in human platelets and plasma have similar mobilities (Erickson and Loskutoff, unpublished observation). The placental PAI[41] and most plasma protease inhibitors[33] migrate in the α region when fractionated by agarose zone electrophoresis. We suggest that this distinction may be used to clarify some of the ambiguities associated with PAIs. The endothelial cell–type PAIs may thus be termed the β-PAIs to distinguish them from these other inhibitors. Obviously, the PAIs in other samples must be analyzed immunologically and by agarose zone electrophoresis to establish this difference as a consistent and useful distinction. These experiments are in progress. Second, the BAE β-PAI is not inactivated by treatment at pH 3 or by incubation in the presence of SDS,[17,42] treatments that rapidly inactivate protease nexin, the placental-derived PAI, and most plasma protease inhibitors. The activities of the BAE β-PAI (Figs. 1–5; ref 22) and, as shown in this report, the human serum β-PAI (Figs 6 to 8; Table 1) are actually stimulated by treatment with SDS and other denaturants, a property that has been used to demonstrate that β-PAIs exist as active and inactive, or latent, forms.[22] Thus, both electrophoretic mobility and biochemical stability

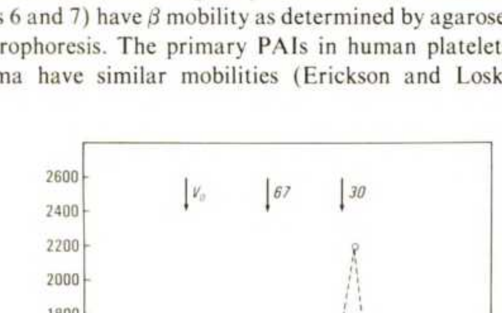

Fig 8. Activation of the PAI in fractions of gel-filtered human serum. DFP-treated human serum (4.5 mL) was chromatographed on Sephacryl S-200 as described in Materials and Methods. The fractions were analyzed in the tPA-binding assay after treatment with dH$_2$O (●), 0.25% SDS (□), or 4 mol/L guanidine-HC1 (○). The arrows indicate the elution positions of blue dextran (mol wt, 2 × 10^6; V$_0$), bovine serum albumin (mol wt, 67,000), and carbonic anhydrase (mol wt, 30,000).

Table 1. SDS Activation of the PAI in BAE CM and in Human Serum

Sample	Dilution	PAI (ng/mL) Control	PAI (ng/mL) SDS
BAE CM	—	3.9	500
	10	8.5	519
Serum sample 1	—	21.8	18.7
	10	25.2	69.0
Serum sample 2	—	9.8	5.4
	10	2.2	59.6

Aliquots of BAE CM or of human whole blood serum from two different donors (samples 1 and 2), were diluted tenfold with PBS/Tw and treated with either dH$_2$O (control) or 0.25% SDS as described in Materials and Methods. The samples were then analyzed in the tPA-binding assay. The results are compared with those obtained with undiluted samples. The data are presented as nanograms of PAI per milliliter of undiluted sample and were obtained by direct comparison with a standard curve constructed using samples of purified BAE PAI.

may be useful parameters for distinguishing between the various PAIs.

In the case of the β-PAI in CM from cultured BAEs, the latent form may represent greater than 95% of the total potential PAI activity. Human endothelial cells and platelets (our unpublished observations) as well as HTC rat hepatoma cells[43] also contain active and latent forms of β-PAI. The latency of the BAE β-PAI seems to be a property of the molecule itself and not the result of the presence of another molecule in CM that masks our ability to detect PAI activity.[22] Whether the PAI is actually synthesized in a latent form or made as an active molecule that is then converted into the latent form either by the cells or upon exposure to the cell culture environment cannot be established from available information. We have shown that the active BAE β-PAI is rapidly inactivated by chloramine T and other oxidants,[44] thus raising the possibility that at least part of the latency of this molecule may result from oxidants in the culture medium. These considerations also may apply to the β-PAI elaborated by other cells or found in platelets, plasma, and serum. The β-PAI in serum was stimulated with SDS only after the serum had been diluted or fractionated chromatographically or electrophoretically (compare Fig 5 with Figs 6 to 8). Since activation is dose-dependent with respect to SDS concentration,[22] we hypothesize that our inability to stimulate the PAI activity of undiluted serum is a consequence of the high protein concentration of serum as compared with CM. These serum proteins may bind the SDS and thus lower its effective concentration with respect to the PAI. The fact that the PAI activity of diluted serum is stimulated by SDS (Table 1) is consistent with this conclusion. The exact mechanism of activation by SDS and guanidine-HCl is not known. However, since these denaturants appear to act directly on the latent PAI,[22] they may induce a conformational change in the molecule that exposes a previously masked reactive site. Activation of plasma/serum β-PAI activity might thus represent an additional means of regulating vascular PA activity.

The results obtained upon gel filtration of serum (Fig 8) revealed three additional characteristics of the serum β-PAI. First, PAI activity was detected in untreated samples only in those fractions that corresponded to a mol wt between 150,000 and 200,000. This is in agreement with the results previously reported by Wiman et al with human plasma.[45] These authors have suggested that this high mol wt component may represent tetrameric PAI or a complex between the PAI and another plasma/serum component. It is not clear why this activity is apparently destroyed by SDS and guanidine. However, this finding is consistent with the fact that no PAI is detected in these fractions by RFA (data not shown). Thus, it is possible that this component may represent a form of active PAI that is SDS sensitive. Others have previously shown that the active PAI in human endothelial cell CM is more sensitive to inactivation by a variety of treatments than is the latent form.[46] Second, both SDS and guanidine-HCl treatments revealed PAI activity in fractions that corresponded to a mol wt of approximately 90,000. This high mol wt form may reflect PAI in complex with another serum component, a complex that is dissociated by denaturants, or may represent aggregates of PAI (Hekman and Loskutoff, unpublished observations). Third, both SDS and guanidine treatments also revealed PAI activity in fractions corresponding to a mol wt of approximately 30,000. This finding is in agreement with our previous report concerning the analysis of the PAI in BAE CM by gel filtration.[22] The difference between this mol wt estimation for the β-PAI and that obtained by SDS-PAGE (ie, 50,000) cannot be fully explained at present. Because most, if not all, of the PAI in serum is derived from platelets,[14] these various mol wt forms of the PAI may also exist in platelets. This possibility is currently being examined.

Although it is not at all clear what function the β-PAI actually serves in normal and pathophysiological situations, the finding of elevated levels of PAI activity in certain individuals with thrombotic disease[15,16] suggests a possible relationship between such conditions and an overall reduction of vascular PA activity. Further examination of this issue awaits the purification of sufficient quantities of human β-PAI.

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REFERENCES

1. Astrup T: Cell-induced fibrinolysis: A fundamental process, in Reich E, Rifkin DB, Shaw E (eds): Proteases and Biological Control. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1975, p 343

2. Collen D: On the regulation and control of fibrinolysis. Thromb Haemost 43:77, 1980

3. Reich E: Activation of plasminogen: A general mechanism for producing localized extracellular proteolysis, in Berlin RD, Hermann H, Lepow IH, Tanzer JM (eds): Molecular Basis of Biological Degradative Processes. Orlando, FL, Academic, 1978, p 155

4. Bachmann F, Kruithof EKO: Tissue plasminogen activator: Chemical and physiological aspects. Semin Thromb Hemost 10:6, 1984

5. Erickson LA, Schleef RR, Ny T, Loskutoff DJ: The fibrinolytic system of the vascular wall. Clin Haematol 14:513, 1985

6. Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator. J Biol Chem 257:2912, 1982

7. Korninger C, Collen D: Neutralization of human extrinsic (tissue-type) plasminogen activator in human plasma: No evidence for a specific inhibitor. Thromb Haemost 46:662, 1981

8. Chmielewska J, Rånby M, Wiman B: Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. Thromb Res 31:427, 1983

9. Verheijen JH, Chang GTG, Kluft C: Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. Thromb Haemost 51:392, 1984

10. Kruithof EKO, Tran-Thang C, Ransijn A, Bachmann F: Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. Blood 64:907, 1984

11. Levin EG: Latent tissue plasminogen activator produced by

human endothelial cells in culture: Evidence for an enzyme-inhibitor complex. Proc Natl Acad Sci USA 80:6804, 1983

12. Thorsen S, Phillips M: Isolation of tissue-type plasminogen activator-inhibitor complexes from human plasma. Evidence for a rapid plasminogen activator inhibitor. Biochim Biophys Acta 802:111, 1984

13. Kruithof EKO, Ransijn A, Tran-Thang C, Bachmann F. Characteristics of a fast-acting inhibitor of plasminogen activator in human plasma. Thromb Haemost 50:193, 1983 (abstr 592)

14. Erickson LA, Ginsberg MH, Loskutoff DJ. Detection and partial characterization of an inhibitor of plasminogen activator in human platelets. J Clin Invest 74:1465, 1984

15. Nilsson IM, Ljungnér H, Tengborn L: Two different mechanisms in patients with venous thrombosis and defective fibrinolysis: Low concentration of plasminogen activator or increased concentration of plasminogen activator inhibitor. Br Med J 290:1453, 1985

16. Hamsten A, Wiman B, de Faire U, Blömback M: Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. New Engl J Med 313:1557, 1985

17. van Mourik JA, Lawrence DA, Loskutoff DJ: Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J Biol Chem 259:14914, 1984

18. Åstedt B, Lecander I, Brodin T, Lundblad A, Löw K: Purification of a specific placental plasminogen activator inhibitor by monoclonal antibody and its complex formation with plasminogen activator. Thromb Haemost 53:122, 1985

19. Scott RW, Bergman BL, Bajpai A, Hersh RT, Rodriquez H, Jones BN, Barreda C, Watts S, Baker JB: Protease nexin. Properties and a modified purification procedure J Biol Chem 260:7029, 1985

20. Erickson LA, Hekman CM, Loskutoff DJ: The primary plasminogen-activator inhibitors in endothelial cells, platelets, serum, and plasma are immunologically related. Proc Natl Acad Sci USA 82:8710, 1985

21. Loskutoff DJ: Effects of acidified fetal bovine serum on the fibrinolytic activity and growth of cells in culture. J Cell Physiol 96:361, 1978

22. Hekman CM, Loskutoff DJ: Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. J Biol Chem 260:11581, 1985

23. Loskutoff DJ: Effect of thrombin on the fibrinolytic activity of cultured bovine endothelial cells. J Clin Invest 64:329, 1979

24. Levin EG, Loskutoff DJ Cultured bovine endothelial cells produce both urokinase and tissue-type plasminogen activators. J Cell Biol 94:631, 1982

25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

26. Erickson LA, Lawrence DA, Loskutoff DJ Reverse fibrin autography: A method to detect and partially characterize protease inhibitors after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Anal Biochem 137:454, 1984

27. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979

28. Johnson DA, Gautsch JW, Sportsman JR, Elder JH Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Techn 1:3, 1984

29. Schleef RR, Sinha M, Loskutoff DJ: An immunoradiometric assay to measure the binding of a specific inhibitor to tissue-type plasminogen activator J Lab Clin Med 106:408, 1985

30. Deutsch DG, Mertz ET. Plasminogen: Purification from human plasma by affinity chromatography. Science 170 1095, 1970

31. Rijken DC, Collen D Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem 256:7035, 1981

32. Fraker PJ, Speck JC Jr. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6 a-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978

33. Müllertz S, Clemmensen I: The primary inhibitor of plasmin in human plasma. Biochem J 159:545, 1976

34. Ny T, Bjersing L, Hsueh AJW, Loskutoff DJ: Cultured granulosa cells produce two plasminogen activators and an antiactivator, each regulated differently by gonadotropins. Endocrinology 116:1666, 1985

35. Mullins DE, Bazer FW, Roberts RM: Secretion of a progesterone-induced inhibitor of plasminogen activator by the porcine uterus. Cell 20:865, 1980

36. Birkedal-Hansen H, Taylor RE: Production of three plasminogen activators and an inhibitor in keratinocyte cultures Biochim Biophys Acta 756:308, 1983

37. Hierowski MT, Schally AV: An inhibitor of urokinase and tissue plasminogen activators in Dunning R3327H prostate tumors of rats treated with D-Trp^6-LH-RH. Horm Res 21:124, 1985

38. Conforti G, Loskutoff DJ. Plasmin and thrombin modulate plasminogen activation by fibrosarcoma cells. Thromb Haemost 54:171, 1985 (abstr 1009)

39. Eaton DL, Baker JB: Evidence that a variety of cultured cells secrete protease nexin and produce a distinct cytoplasmic serine protease-binding factor. J Cell Physiol 117:175, 1983

40. Seifert SC, Gelehrter TD: Mechanism of dexamethasone inhibition of plasminogen activator in rat hepatoma cells. Proc Natl Acad Sci USA 75:6130, 1978

41. Kawano T, Morimoto K, Uemura Y: Partial purification and properties of urokinase inhibitor from human placenta. J Biochem 67:333, 1970

42. Loskutoff DJ, van Mourik JA, Erickson LA, Lawrence DA. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. Proc Natl Acad Sci USA 80:2956, 1983

43. Loskutoff DJ, Roegner K, Erickson LA, Schleef RR, Huttenlocher A, Coleman PL, Gelehrter TD: The dexamethasone-induced inhibitor of plasminogen activator in hepatoma cells is antigenically related to an inhibitor produced by bovine aortic endothelial cells. Thromb Haemost 55 8, 1986

44. Lawrence D, Curriden S, Loskutoff DJ: Selective inactivation of the rapidly acting plasminogen activator inhibitor (PAI) by mild oxidation. Thromb Haemost 54:229, 1985 (abstr 1358)

45. Wiman B, Chmielewska J, Rånby M: Inactivation of tissue plasminogen activator in plasma. Demonstration of a complex with a new rapid inhibitor. J Biol Chem 259:3644, 1984

46. Sprengers ED, Verheijen JH, Van Hinsbergh VWM, Emeis JJ: Evidence for the presence of two different fibrinolytic inhibitors in human endothelial cell conditioned medium. Biochim Biophys Acta 801:163, 1984

Shifts in Blast Cell Phenotype and Karyotype at Relapse of Childhood Lymphoblastic Leukemia

By Ching-Hon Pui, Susana C. Raimondi, Frederick G. Behm, Judith Ochs, Wayne L. Furman,
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Analyses of bone marrow blast cells collected at diagnosis and relapse from 68 children with acute lymphoblastic leukemia (ALL) demonstrated changes in the expression of cell markers in one-fourth of the patients. Loss of the common ALL antigen (CALLA) was a frequent change, occurring in 8 of the 51 cases initially classified as common or pre-B ALL. The HLA-DR antigen was either acquired or lost in 5 of the 68 cases, terminal deoxynucleotidyl transferase was lost in 6 of 25 cases, and reactivity of the T10 antigen with monoclonal antibodies was increased in 6 of 17 cases of non-T cell ALL. Conversion to acute nonlymphoblastic leukemia, so-called lineage switch, was noted in two cases of common ALL and one of pre-B ALL, coinciding with the loss of CALLA. Results of chromosomal analyses in cases with a loss of CALLA implicated several mechanisms in the observed phenotypic changes. In six cases, including each instance of lineage switch, the original karyotype had been replaced by an entirely different abnormal karyotype, suggesting clonal selection or induction of a second malignancy. In another case, the evidence suggested clonal evolution. Our findings demonstrate that sequential phenotypic and cytogenetic studies may yield valuable insights into the mechanisms of leukemic recurrence and may have implications for treatment selection.
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DETERMINING blast cell biological features at diagnosis of acute lymphoblastic leukemia (ALL) has clinical relevance in the context of cell classification schemes and particular treatment programs. Numerous studies have demonstrated close relationships between the outcome of therapy and blast cell morphology, surface immunophenotype, number of glucocorticoid receptor sites per cell and, more recently, chromosomal abnormalities (see ref. 1 for review). Repeated assessment of selected markers at later intervals in the clinical course might be expected to contribute important information for understanding mechanisms of disease recurrence or for planning alternative therapy. Most previous studies have indicated that blast cell phenotypes remain stable over time, with only minor changes occurring in a small proportion of patients.[2-5] With the availability of comprehensive assays for lymphoid and myeloid cell markers, however, an increasing number of reports have claimed major phenotypic shifts in ALL.[6-8]

We had the opportunity to study 68 cases of ALL at both diagnosis and relapse to document possible changes in lymphoblast phenotype. The results indicate a relatively high frequency of phenotypic shift in ALL, mainly disappearance of the common ALL antigen (CALLA). Results of cytogenetic studies suggest that clonal evolution, clonal selection, or the emergence of an independent clone is responsible for most of these changes.

MATERIALS AND METHODS

One hundred twenty-seven children with ALL who were admitted to Total Therapy Study X[9] at St Jude Children's Research Hospital experienced their first hematologic relapse between September 1980 and October 1985. Sixty-eight patients had adequate bone marrow samples for blast cell immunophenotyping at both diagnosis and relapse and are the subjects of this report Informed written consent was obtained from the patients, their parents, or both.

Immunologic cell typing. Bone marrow cells were separated on a Ficoll-Hypaque gradient, and only samples containing >85% blasts were assayed They were considered positive for heat-stable E rosette formation (E) 37 °C if at least 2% of the blast cells formed rosettes. Cell-surface antigens were detected by a standard indirect immunofluorescence assay with monoclonal antibodies representative of the major cluster groups (CD) identified at the First and Second International Workshops on Human Leukocyte Differentiated Antigens. They included J5 (CALLA, or CD-10),[10], HLA-DR, T10,[11] and T cell-specific antigens (T)-T101 (CD-5),[12] T3 (CD-3),[13] and T11 (CD-2)[14] Two investigators independently counted 200 cells in each immunofluorescence assay; the results were judged positive if ≥40% of the blast cells had surface fluorescence Cell-surface immunoglobulin (SIg) was identified by direct fluorescence with fluoresceinated goat anti-human immunoglobulin Cytoplasmic immunoglobulin (CIg) was identified by a direct method that used fluoresceinated $F(ab')_2$ goat anti-human μ-chains (Southern Biotechnology, Birmingham, AL);[15] positive cases were those in which ≥10% blast cells contained CIg. Depending on their pattern of reactivity, the samples were classified phenotypically as common ALL (CALLA$^+$, HLA-DR$^+$, E$^-$, T$^-$, CIg$^-$, SIg$^-$), T cell (T$^+$, E rosette$^\pm$, HLA-DR$^-$), pre-B cell (CIg$^+$, HLA-DR$^\pm$, CALLA$^\pm$), or CALLA$^-$ (HLA-DR$^+$, CALLA$^-$, CIg$^-$, SIg$^-$, T$^-$). Terminal deoxynucleotidyl transferase (TdT) was identified by use of indirect immunofluorescence[16] Cells from selected patients were examined for myeloid-associated surface antigens. My-1 (X-Hapten, CD-15),[17] MCS.2 (CDw-13),[18] 5F1 (CDw-14),[19] Mol (CD-11),[20] and SJ-D1 (gp150)[21]

Cytochemical studies. Bone marrow smears were stained by standard techniques including periodic acid-Schiff reagent, Sudan black B, myeloperoxidase, naphthol AS-D chloroacetate esterase, and α-naphthyl butyrate esterase. The presence of >3% positive

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blast cells was considered a positive result. Morphologic classification followed French-American-British (FAB) conventions.[22]

Cytogenetic studies Bone marrow samples were prepared by a direct technique developed for ALL,[23] and metaphases were G-banded by a modified Trypsin method.[24]

RESULTS

Of the 68 patients who were studied sequentially, 39 had common ALL at diagnosis, 16 had pre-B (12 CALLA$^+$), 8 had CALLA$^-$, and 5 had T cell ALL. Although the number of cell marker studies performed for each sample varied according to the number of leukemic blast cells available, tests for CALLA and HLA-DR were performed in every case.

Shifts in antigen expression at relapse are shown in Table 1. A frequent alteration involved the loss of CALLA in five cases of common ALL and in three of pre-B ALL. In no instance did blast cells acquire CALLA after being negative for the antigen at diagnosis. The HLA-DR antigen was lost in 1 pre-B and 2 common ALL cases, but was acquired in 1 of 3 common and 1 of 3 pre-B cases that originally lacked this marker. TdT activity was evident at diagnosis in all but 2 of the cases tested (both common ALL), decreasing to negligible levels in 3 of 10 cases of common ALL and 3 of 10 pre-B cases that were examined at relapse. TdT activity remained positive in the three CALLA$^-$ cases tested. The T10 antigen was detected at diagnosis on blast cells in 10 of the 17 non-T cases that were tested, appearing at relapse in 6 of the 7 T10-negative cases.

Loss of CALLA was associated with conversion to acute nonlymphoblastic leukemia (ANLL) in two cases that initially had common ALL and in one patient with CALLA$^+$ pre-B leukemia. The immunologic and cytochemical studies of blast cells from these three cases are summarized in Table 2. Besides the loss of CALLA, both tested cases lacked TdT activity, and CIg was no longer detectable in the case initially classified as pre-B Notably, myeloid-associated surface antigens were present at diagnosis on the blast cells from patient 1. At diagnosis, the leukemic cells from all three patients met stringent morphologic criteria for ALL and lacked cytochemical evidence of myeloid or monocytic features. When examined at relapse, the cells reacted with myeloid-associated cytochemical stains, and Auer rods were present in case 4.

The results of sequential cytogenic studies in the eight cases showing a loss of CALLA are presented in Table 3 Evidence for a different leukemic clone at relapse was obtained in six of the cases. The original blast cell karyotype of cases that later converted to ANLL was replaced by an entirely different karyotype. At the time of lineage switch, each case had a pseudodiploid karyotype and a different translocation involving the long arm of chromosome 11 at band q23 (11q23). The other three cases, which had converted from common to CALLA$^-$ ALL, also had different pseudodiploid karyotypes at relapse, characterized by a translocation involving 11q23 in the two with complete banding study. Of the remaining two cases, one (patient 13) showed evidence of marked clonal evolution, and the other (patient 12) retained the apparent constitutional abnormality.

DISCUSSION

Seventeen (25%) of the 68 patients in this series had blast cell phenotypic shifts at relapse. Loss of the common ALL antigen was a frequent change, occurring in 16% of cases that were clearly CALLA$^+$ at diagnosis. Greaves and colleagues[3,4] also demonstrated a loss of CALLA in similar

Table 1. Shifts in Blast Cell Differentiation Markers

Case	At Diagnosis			Positivity at Diagnosis → Relapse (%)				
	Age (yr)	WBC (× 10^9/L)	Immunophenotype	CALLA	HLA-DR	TdT	T10	Other Markers
1	8	3.3	Common	96 → 6	—	96 → 0	2 → 53	—
2	2	27.8	Common	46 → 4	—	95 → 25	ND	—
3*	16	28.8	Common	64 → 10	78 → 18	ND	ND	—
4	3	7.4	Common	76 → 3	—	ND	ND	—
5	2	11.5	Common	90 → 8	—	—	ND	—
6	3	4.3	Common	—	22 → 72	94 → 17	ND	—
7	3	4.2	Common	—	71 → 14	ND	ND	—
8	4	24.4	Common	—	—	—	24 → 47	—
9	6	10.1	Common	—	—	—	30 → 65	—
10	5	2.8	Common	—	—	—	1 → 59	—
11	3	8.4	Pre-B	70 → 24	—	91 → 2	—	CIg (83 → 3)
12	2	35	Pre-B	74 → 10	67 → 18	—	—	—
13	3	13	Pre-B	69 → 4	—	ND	ND	—
14	7	13.7	Pre-B	—	6 → 50	93 → 0	—	—
15	14	345	Pre-B	—	—	84 → 6	—	—
16	7	26.7	CALLA$^-$	—	—	—	34 → 70	—
17	14	88	CALLA$^-$	—	—	—	26 → 76	—

WBC, white blood cell count, CALLA, common ALL antigen; TdT, terminal deoxynucleotidyl transferase; CIg, cytoplasmic immunoglobulin, ND, not done

Dash indicates that results were similar at diagnosis and relapse

*Cytoplasmic immunoglobulin was not tested at diagnosis and was not detectable at relapse.

Table 2. Laboratory Findings in Cases of Lineage Switch

	Case 1*		Case 4		Case 11	
	Diagnosis	LS	Diagnosis	LS	Diagnosis	LS
Immunological markers†						
Common ALL antigen	96	6	76	3	70	24
HLA-DR	86	84	35	52	78	93
E rosette formation	0	0	ND	ND	0	0
T101	4	19	ND	ND	ND	ND
Cytoplasmic immunoglobulin	0	0	ND	ND	84	3
Terminal transferase	96	0	ND	ND	91	2
My-1	68	22	ND	ND	ND	60
MCS-2	44	66	ND	ND	ND	55
5F1	39	38	ND	28	ND	44
Mol	48	26	ND	21	ND	68
SJ-D1	54	64	ND	ND	ND	57
Cytochemical stains†						
Periodic acid-Schiff	+	ND	+	–	–	–
Sudan black B	–	ND	–	ND	–	20
Myeloperoxidase	–	5	–	+	–	8
Chloroacetate esterase	–	ND	–	ND	–	–
α-Naphthyl butyrate esterase	–	56	—	ND	—	84
FAB subtype	L1	M5	L1	M2‡	L1	M5

LS, lineage switch, ALL, acute lymphoblastic leukemia; ND, not done, FAB, French-American-British; +, positive, –, negative

*Presence of myeloid-associated surface antigens at diagnosis.

†Results given as percentages of positive cells.

‡Auer rods present.

proportions of cases studied at diagnosis and relapse; however, there is little information regarding the significance of changes in CALLA expression at relapse.

Of the 8 cases that converted from CALLA$^+$ to CALLA$^-$, 3 showed lineage switch to ANLL, , 2 were refractory to retreatment, and 3 responded to therapy; their reinduced remissions were short, however (2, 5, and 10 months, respectively). In contrast, 12 of the 43 patients who retained CALLA at relapse remain in second remission for 7+ to 56+ months (median, 23+ months) Aside from the length

Table 3. Cytogenetic Findings in Cases With a Loss of CALLA

Case No	Time to Relapse (mo)	At Diagnosis		At Relapse	
		Immunophenotype	Karyotype	Immunophenotype	Karyotype
1	26	Common ALL	47,XY,+9	ANLL (M5)	46,XY,–18,+der(18),t(18;?)(p11 3;?),t(9,11)(p21,q23)/46,XY,t(9;11)(p21;q23
/46,XY,t(9;11)(p21;q23)					
2	37	Common ALL	46,XX,–20,t(2;16)(p13;p13)/45,XX,–20,–22,+mar,t(2,16)(p13;p13)	CALLA$^-$ ALL	46,XX,t(4;11)(q21;q23)
3	10	Common ALL	46,XX,–19,+der(19),t(1;19)(q23;p13 3)	CALLA$^-$ ALL	46,XX,mar*
4	69	Common ALL	54,XY,+X,+5,+6,+10,+11,+D.+21,del(7)(q22)	ANLL (M2)	46,XY,t(11;17)(q23,q21)
5	16	Common ALL	53,XY,+X,+6,+9,+15,+17,+21,+mar	CALLA$^-$ ALL	46,XY,t(11,19)(q23,p13)
11	36	Pre-B ALL	46,XY,–12,+der(12),t(12,?)(p12;?)	ANLL (M5)	46,XY,t(11,21)(q23;q22)
12	9	Pre-B ALL	46,XY,t(1;19)(q23;p13.3),t(9;14)(q13,p11),del(13)(q21)/46,XY,inv(9)(p11q13)†	Pre-B ALL	46,XY,inv(9)(p11q13)†
13	27	Pre-B ALL	46,XY,–13,+der(13),t(13;?)(q34;?),t(1;19)(q23;p13 3)	Pre-B ALL	85 chromosomes with t(1;19)(q23;p13.3) and dup(14)(q13q32) but variable numerical abnormalities

CALLA, common ALL antigen; ALL, acute lymphoblastic leukemia, ANLL, acute nonlymphoblastic leukemia.

*Banding preparation was inadequate for complete interpretation. The marker is a metacentric chromosome of group A.

†The inv(9) was interpreted as a constitutional abnormality that occurs commonly in the general population.

of a patient's initial hematologic remission,[25,26] there are no reliable predictors of outcome following a bone marrow relapse. Our data suggest that loss of CALLA is a poor prognostic sign in such cases. Despite the relatively long initial periods of hematologic remission in several of our CALLA^{+} → CALLA^{-} patients (Table 3), ultimate outcome in this group has been poor. The apparent adverse prognostic effect of CALLA loss from leukemic cells should be interpreted with some caution, however, as our study population was relatively small, had been treated in several different protocols for relapsed leukemia, and had variable durations of initial hematologic remission.

Acquisition or loss of the HLA-DR antigen, noted in five of our patients, has been observed in previous studies.[2-5] Loss of blast cell TdT activity was also a common finding. In two of the six patients tested, decreased TdT activity at relapse was associated with lineage switch. Additional studies are needed to determine the significance of this alteration. Blast cells from 10 of 17 patients with non-T ALL showed T10 positivity at diagnosis; in the other 6 cases, the antigen was acquired at relapse, confirming reports that T10 is not restricted to cells of T lineage.[11]

The molecular basis for aberrant regulation of lineage-specific differentiation antigens in ALL is unknown; however, the karyotypes available for cases showing a loss of CALLA (Table 3) suggest several mechanisms of phenotypic shift at the chromosomal level. We think it remarkable that in cases 1 through 5 and 11, the original karyotype was replaced by an entirely different abnormal karyotype, suggesting induction of a second malignancy or perhaps selection and overgrowth of an independent drug-resistant clone that could not be detected at diagnosis. We favor the first explanation because of the relatively long intervals to relapse, 2 to 5 years, in the three cases that converted to ANLL, and the lack of evidence for separate populations of myeloid and lymphoid leukemic cells coexisting in the same patient at diagnosis. At relapse, each of the cases with complete banding analyses possessed a chromosomal translocation involving 11q23, a region commonly affected by cytogenetic changes in the myelomonocytic subtype of ANLL,[27] CALLA^{-} ALL,[28] and leukemia with mixed-lineage expression.[29,30] It has been suggested that pluripotent stem cells are transformed in leukemia with 11q23 involvement, and that phenotypic expression is influenced by genes from the reciprocally translocated chromosome.[28,31] Thus, loss of CALLA in these cases may be related to malignant transformation of a pluripotent stem cell after eradication of the original B cell-precursor and pre-B stem line by chemotherapy. The c-*ets*-1 oncogene was mapped to the 11q23 region and may be involved in the pathogenesis of acute leukemias, with a translocation affecting this locus.[32] Indeed, Rovigatti and colleagues[33] recently demonstrated amplification and rearrangement of this oncogene in a case of acute myelomonocytic leukemia and a case of small lymphocytic cell lymphoma with 11q23 involvement. Case 12 cannot be interpreted because of failure to demonstrate a malignant stem line at relapse. Case 13 represents the only instance in which karyotypic findings at diagnosis were retained in the reemergent blast cells (clonal evolution).

Although not observed in the T cell cases in this series, lineage switch has occurred in childhood leukemia of thymic origin. Stass and co-workers,[6] for example, showed conversion to ANLL in five cases that were initially classified as T cell ALL. Early recognition of lineage switch may be helpful in selecting an effective plan of retreatment. Each of the three patients who had undergone conversion to myeloid leukemia in this study achieved a second remission with ANLL-directed induction therapy. Finally, monoclonal antibodies with complement or those conjugated to ricin have been used to purge bone marrow before autologous transplantation [34,35] For such therapeutic approaches, it is essential to have an accurate profile of antigen expression at relapse.

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REFERENCES

1 Kalwinsky DK, Roberson P, Dahl G, Harber J, Rivera G, Bowman WP, Pui C-H, Ochs J, Abromowitch M, Costlow ME, Melvin SL, Stass S, Williams DL, Murphy SB Clinical relevance of lymphoblast biological features in children with acute lymphoblastic leukemia J Clin Oncol 3 477, 1985

2 Borella L, Casper JT, Lauer SJ: Shifts in expression of cell membrane phenotypes in childhood lymphoid malignancies at relapse. Blood 54 64, 1979

3. Greaves M, Paxton A, Janossy G, Pain C, Johnson S, Lister TA: Acute lymphoblastic leukaemia associated antigen. III. Alterations in expression during treatment and in relapse. Leuk Res 4 1, 1980

4. Greaves MF. Analysis of the clinical and biological significance of lymphoid phenotypes in acute leukemia. Cancer Res 41.4752, 1981

5. Lauer S, Piaskowski V, Camitta B, Casper J. Bone marrow and extramedullary variations of cell membrane antigen expression in childhood lymphoid neoplasias at relapse. Leuk Res 6.769, 1982

6. Stass S, Mirro J, Melvin S, Pui C-H, Murphy SB, Williams D· Lineage switch in acute leukemia Blood 64·701, 1984

7 Murphy SB, Stass SA, Kalwinsky D, Rivera G· Phenotypic conversion of acute leukemia from T-lymphoblastic to myeloblastic induced by therapy with 2′-deoxycoformycin. Br J Haematol 55·285, 1983

8. Hershfield MS, Kurtzberg J, Harden E, Moore JD, Whang-Peng J, Haynes BF: Conversion of a stem cell leukemia from a T-lymphoid to myeloid phenotype induced by adenosine deaminase inhibitor 2′-deoxycoformycin Proc Natl Acad Sci USA 81:253, 1984

9. Pui C-H, Dahl GV, Bowman WP, Rao BN, Abromowitch M, Ochs J, Rivera G: Elective testicular biopsy during chemotherapy for childhood leukaemia is of no clinical value Lancet 2·410, 1985

10 Ritz J, Pesando JM, Notis-McConarty J, Lazarus H, Schlossman SF A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature 283·583, 1980

11. Terhorst C, van Agthoven A, LeClair K, Snow P, Reinherz E, Schlossman S. Biochemical studies of the human thymocyte cell-surface antigens T6, T9 and T10. Cell 23 771, 1981

12. Royston I, Majda JA, Baird SM, Meserve BL, Griffiths JC. Human T-cell antigens defined by monoclonal antibodies The

65,000-dalton antigen of T-cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. J Immunol 125:725, 1980

13. Reinherz EL, Kung PC, Goldstein G, Schlossman SF: Separation of functional subsets of human T cells by a monoclonal antibody Proc Natl Acad Sci USA 76 4061, 1979

14. Verbi W, Greaves MF, Schneider C, Koubek K, Janossy G, Stein H, Kung P, Goldstein G: Monoclonal antibodies OKT 11 and OK T11A have pan-T reactivity and block sheep erythrocyte "receptors." Eur J Immunol 12:81, 1982

15. Volger LB, Crist WM, Bockman DE, Pearl ER, Lawton AR, Cooper MD· Pre-B-cell leukemia· A new phenotype of childhood lymphoblastic leukemia. N Engl J Med 298.872, 1978

16 Stass SA, Schumacher HR, Keneklis TP, Bollum FJ. Terminal deoxynucleotidyl transferase immunofluorescence of bone marrow smears Experience in 156 cases. Am J Clin Pathol 72.898, 1979

17. Civin CI, Mirro J, Banquerigo ML. My-1, a new myeloid-specific antigen identified by a mouse monoclonal antibody. Blood 57:842, 1981

18. Gregg SL, Gajl-Peczalska KJ, LeBien TW, Bloomfield CD, Brunning R, Sagawa K. Monoclonal antibody MSC-2 as the marker of phorbol diester-induced myeloid differentiation in acute undifferentiated leukemia. Cancer Res 44:2724, 1984

19. Bernstein ID, Andrews RG, Cohen SF, McMaster BE: Normal and malignant human myelocytic and monocytic cells identified by monoclonal antibodies. J Immunol 128.876, 1982

20 Todd RF III, Griffin JD, Ritz J, Nadler LM, Abrams T, Schlossman SF: Expression of normal monocytic-macrophage differentiation antigens on HL60 promyelocytes undergoing differentiation induced by leukocyte-conditioned medium or phorbol diester. Leuk Res 5.491, 1981

21. Look AT, Peiper SC, Rebentisch MB, Ashmun RA, Roussel MF, Rettenmeier CW, Sherr CJ: Transfer and expression of the gene encoding a human myeloid membrane antigen (gp150). J Clin Invest 75·569, 1985

22 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposals for the classifications of the acute leukaemias Br J Haematol 33:451, 1976

23 Williams DL, Harris A, Williams KJ, Brosius MJ, Lemonds W: A direct bone marrow chromosome technique for acute lymphoblastic leukemia. Cancer Genet Cytogenet 13:239, 1984

24. Seabright M. A rapid banding technique for human chromosomes. Lancet 2:971, 1971

25 Chessells J, Leiper A, Rogers D: Outcome following late marrow relapse in childhood acute lymphoblastic leukemia J Clin Oncol 2:1088, 1984

26 Rivera GK, Buchanan G, Boyett JM, Camitta B, Ochs J, Kalwinsky D, Amylon M, Vietti TJ, Crist WM. Intensive retreatment of childhood acute lymphoblastic leukemia in first bone marrow relapse. A Pediatric Oncology Group study. N Engl J Med 315.273, 1986

27 Fourth International Workshop on Chromosomes in Leukemia 1982: Rearrangements of 11q. Cancer Genet Cytogenet 11:294, 1984

28 Kaneko Y, Maseki N, Takasaki N, Sakurai M, Hayashi Y, Nakazawa S, Mori T, Sakurai M, Takeda T, Shikano T, Hiyoshi Y: Clinical and hematologic characteristics in acute leukemia with 11q23 translocations Blood 67 484, 1986

29 Crist WM, Cleary ML, Grossi CE, Prasthofer EF, Heggie GD, Omura GA, Carroll AJ, Link MP, Sklar J: Acute leukemias associated with the 4;11 chromosome translocation have rearranged immunoglobulin heavy chain genes Blood 66:33, 1985

30 Mirro J, Kitchingman G, Williams D, Lauzon GJ, Lin C-C, Callihan T, Zipf TF: Clinical and laboratory characteristics of acute leukemia with the 4;11 translocation. Blood 67·689, 1986

31. Abe R, Sandberg AA· Significance of abnormalities involving chromosomal segment 11q22–25 in acute leukemia. Cancer Genet Cytogenet 13.121, 1984

32. de Taisne C, Gegonne A, Stehelin D, Bernheim A, Berger R· Chromosomal localization of the human proto-oncogene c-ets. Nature (Lond) 310:581, 1984

33. Rovigatti U, Watson DK, Yunis JJ. Amplification and rearrangement of Hu-ets-1 in leukemia and lymphoma with involvement of 11q23. Science 232:398, 1986

34 Ritz J, Schlossman SF: Utilization of monoclonal antibodies in the treatment of leukemia and lymphoma. Blood 59:1, 1982

35. Foon KA, Schroff RW, Gale RP· Surface markers on leukemia and lymphoma cells· Recent advances Blood 60:1, 1982

Autologous Bone Marrow Transplantation in Acute Myelogenous Leukemia: In Vitro Treatment With Myeloid Cell-Specific Monoclonal Antibodies

By Edward D. Ball, Letha E. Mills, Christopher T. Coughlin, J. Robert Beck, and Gibbons G. Cornwell III

Second or third chemotherapy-induced remissions in acute myelogenous leukemia (AML) are limited by early relapse of the leukemia. We developed monoclonal antibodies (MoAbs) that are cytotoxic to myeloid leukemia cells to treat bone marrow from these patients ex vivo for autologous transplantation. In this pilot study, bone marrow was harvested from ten patients with AML in remission, treated with one or two complement-fixing MoAbs, PM-81 and AML-2-23, which react with myeloid differentiation antigens, incubated with rabbit complement, and cryopreserved. These MoAbs were chosen because they have broad reactivity with AML cells but not with pluripotent progenitor cells. At the time of transplant, 6 patients were in second complete remission, 1 each was in third complete or partial remission, and 2 were in early first relapse. The patients were treated with cyclophosphamide (60 mg/kg a day for 2 days) and total body irradiation (200 cGy twice a day for 3 days) and given infusions of MoAb-treated bone marrow. Full bone marrow reconstitution was observed in eight patients; two patients did not recover platelets. Seven of the ten patients are surviving and disease-free at 21.0, 15.0, 13.0, 10.0, 6.0, 3.0, and 2.0 months posttransplant. Treating bone marrow with MoAbs to myeloid differentiation antigens does not interfere with pluripotential stem cell engraftment. Longer follow-up and a controlled study are necessary to prove that the apparent efficacy of this therapeutic approach in some patients is attributable to MoAb-mediated killing of leukemia cells.

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TREATMENT OF ACUTE myelogenous leukemia (AML) with combination chemotherapy results in a complete remission (CR) in 50% to 80% of patients.[1] Most patients relapse and ultimately succumb to their disease, however. Therefore, improved strategies for the elimination of any blast cells remaining at the time of CR are urgently needed.

Bone marrow transplantation during remission of AML is being performed primarily using HLA-identical or syngeneic donors.[2-4] Although encouraging results have been reported, this approach is generally limited to younger patients who have HLA-matched donors. Therefore, new approaches to transplantation in AML, such as using monoclonal antibodies (MoAbs) to purge marrow of leukemic cells,[5] might allow wider use of this technique, possibly with less toxicity.

We recently described a panel of complement-fixing MoAbs that are specifically reactive with myeloid cells[6,7] and recognize antigens expressed on AML blast cells.[7-11] One of these MoAbs, PM-81, binds with high affinity to a family of glycolipids and glycoproteins sharing a common terminal pentasaccharide, lacto-*N*-fucopentaose-III.[12] This MoAb binds to blast cells from >90% of patients with AML regardless of French-American-British (FAB) subclass.[8,11] Another MoAb, AML-2-23, binds to a glycoprotein antigen of 55,000 daltons.[13] AML-2-23 reacts primarily with blasts from patients with myelomonocytic or monocytic leukemia.[12] MoAbs PM-81 and AML-2-23 react to some extent with granulocyte/monocyte colony-forming cells (CFU-GM) but not erythroid (BFU-E) or mixed (CFU-GEM and CFU-GEMM) protenitors.[14] In a recent study performed by the Cancer and Leukemia Group B, PM-81 and AML-2-23 were reactive with cells from 91% and 77% of patients with AML; the first and third (after anti-HLA-DR) highest percentages were obtained using a panel of ten antimyeloid MoAbs.[8] In addition, the majority of clonogenic leukemia cells from two-thirds of patients studied expressed one or both of the antigens defined by the MoAbs.[14] We have begun to use MoAbs AML-2-23 and PM-81 to treat bone marrow harvested from patients with AML in remission in preparation for subsequent autologous bone marrow transplantation. This report describes our experience with the first ten patients and demonstrates the feasibility of this therapy.

MATERIALS AND METHODS

Monoclonal antibody purification. MoAbs PM-81 and AML-2-23 were purified from ascites as previously described.[15] All MoAb preparations were tested for sterility by culture in trypticase soy broth and for endotoxin by the Limulus amoebocyte lysate assay [15]

Surface marker analysis of leukemia cells. Blast cells obtained from bone marrow at the time of diagnosis or first relapse were separated by Ficoll-Hypaque gradient centrifugation as described[9] and incubated with purified MoAb (100 μl at 20 μg/mL) for 60 minutes at 4° C in the presence of human IgG (10^{-5} mol/L) to block Fc receptor binding After cells were washed with phosphate-buffered saline (PBS) (pH 7.4) containing bovine serum albumin (BSA) (0.1%) and sodium azide (AZ) (0.5%), a fluorescein-isothiocyanate-labeled goat anti-mouse Ig F(ab')$_2$ antibody (Boehringer-Mannheim, Indianapolis) was added for a further incubation of 30 minutes at 4° C. After being washed with PBS/BSA/AZ, the cells were fixed with 2% paraformaldehyde and analyzed on the Ortho (Westwood, MA) Cytofluorograf system 50H with the 2150 computer system

Complement-mediated cytotoxicity. Cytotoxicity of MoAbs for leukemia cells was assessed by incubation of cells with MoAbs (as above) followed by the addition of neonatal rabbit serum (Pel Freez, Rogers, AR) to achieve a final dilution of 1:6 After being incubated

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for 1 hour at 22° C with continuous mixing, the cells were washed and their viability was assessed using acridine orange and ethidium bromide.[16]

Processing and treating of bone marrow cells An average of 6.3×10^8 (SE 0.8×10^8, range 3.5 to 11.5×10^8) nucleated bone marrow cells per kilogram of body weight were removed through multiple punctures of the anterior and posterior iliac crests of patients under general anesthesia and collected in Medium-199 (GIBCO, Grand Island, NY) containing preservative-free heparin (Sigma Chemical Co, St Louis). Mononuclear cells were separated by centrifugation on a Haemonetics (Braintree, MA) Model 30 cell processor[17] with a mean recovery of 1.5×10^8 (SE − 0.47×10^8, range 0.4 to 7.6×10^8) cells per kilogram of body weight. The cells were incubated on a platform shaker with purified MoAbs PM-81 (50 µg/mL) alone (patients 4 through 6) or with MoAbs PM-81 and AML-2-23 (both at 50 µg/mL) (patients 1 through 3 and 7 through 10) for 15 minutes, followed by the addition of neonatal rabbit serum to achieve a final dilution of 1:6.[18] Deoxyribonuclease Type I (10 U/mL) (Sigma) was added to the cell suspension to decrease the tendency of cells to aggregate following lysis After 60 minutes at room temperature, the cells were sedimented at 500 g, and the supernatant was removed This process was repeated, and the cells were assessed for viability and adjusted to 8×10^7 cells/mL with Medium-199 The mean recovery of cells after MoAb treatment was 2.6×10^7) (SE − 0.34×10^7, range 1.8 to 5.4×10^7) cells per kilogram of body weight. A solution of Medium-199 containing 20% dimethylsulfoxide (Rimso-100, Research Industries Corporation, Salt Lake City) and 10% irradiated (1,500 rad) autologous plasma was added in equal volume to the cell suspension, which was then aliquoted into sterile freezing bags (Delmed Inc, Canton, MA) The cells were frozen at a rate of 3° C/min to −80° C using a controlled-rate freezer (Union Carbide, Indianapolis) and transferred to liquid nitrogen for storage

Effect of MoAb + complement treatment on granulocyte/monocyte progenitor cells. The ability of the patients' bone marrow cells before and after MoAb therapy to form colonies in methylcellulose cultures in the presence of colony-stimulating factor was measured as previously described[9] After 14 days, CFU-GM were counted on an inverted microscope

Ablative therapy and autologous bone marrow transplantation (ABMT) Cytoreductive therapy consisted of cyclophosphamide (60 mg/kg body weight on days −5 and −4), followed by total body irradiation (TBI) (1,200 cGy total in twice-daily doses 6 hours apart of 200 cGy on days −3, −2, and −1) delivered with a Cobalt-60 source by opposed lateral fields without lung shielding.[19] On day 0, frozen bone marrow was quickly thawed at 38° C and administered through a central venous line All patients received intrathecal methotrexate (12 mg) on day −3

Patient characteristics Patients ranged in age from 16 to 44 years and included six female and four male patients (Table 1). Five patients had their marrow harvested in first CR, four had their marrow harvested in second CR, and one had marrow harvested in third CR Transplantation was performed after a second CR was achieved in three patients (nos. 4, 8, and 10) previously harvested in first CR and at early relapse (9% myeloblasts in the bone marrow in patient 6 and a breast chloroma in patient 7) in two other patients harvested in first CR. Three patients (nos 1, 2, and 9) harvested in second CR were also transplanted in second CR. One patient (no 5) whose marrow was harvested in second CR relapsed before transplant could be performed. After an attempt was made to reinduce remission with homoharringtonin, his bone marrow biopsy was hypocellular and contained a small focus of blasts at the time of ABMT The patient (no 3) whose marrow was harvested in third remission was transplanted in third remission Informed consent was obtained for all aspects of the bone marrow transplant following guidelines approved by the Committee for the Protection of Human Subjects of the Dartmouth-Hitchcock Medical Center.

Table 1. Nos. of CFU-GM Before and After MoAb Treatment

	CFU-GM/10^5 Cells		Tota CFU-GM*		
Patient	Before	After	Before	After	% Change
1	42	78	7.98×10^4	2.03×10^4	−75
2	22	26	1.61×10^4	0.68×10^4	−58
3	20	60	1.04×10^4	1.08×10^4	0
4	14	18	10.6×10^4	0.56×10^4	−95
5	22	30	2.64×10^4	1.62×10^4	−38
6	30	45	2.64×10^4	1.08×10^4	−59
7	32	70	2.24×10^4	1.40×10^4	−37
8	18	35	0.72×10^4	$.77 \times 10^4$	0
9	26	53	1.6×10^4	0.98×10^4	−39
10	37	133	1.6×10^4	2.4×10^4	+50
	Average		$3.26 \pm$ 1.04×10^4	$1.36 \pm$ $.25 \times 10^4$	

MoAb, monoclonal antibody

Quadruplicate cultures of bone marrow cells before and after MoAb + C′ treatment were performed in methylcellulose cultures using giant cell tumor conditioned medium (GCT-CM) as a source of colony-stimulating activity Granulocyte/monocyte colonies of ≥40 cells were counted by microscopy on day 14 of culture in a humidified atmosphere of 5% CO_2.

*Absolute number of CFU-GM per kilogram of body weight was derived by multiplication of CFU-GM concentration by numbers of mononuclear cells before and after MoAb treatment

All patients had received cytosine arabinoside (ARA-C) and daunorubicin (DNR) as their initial remission induction chemotherapy Reinduction therapy for patients 1 through 3 consisted of ARA-C (patient 1 received 3 g/m² every 12 hours for 2 days; patients 2 and 3 received 100 mg/m² a day for 7 days) Patients 4, 8, and 10 achieved CR with mitoxantrone Patient 5 was treated with homoharringtonin at second relapse Patient 6 was transplanted at the first sign of bone marrow relapse after 20 months of remission following ARA-C/DNR induction and postremission intensification. Patient 7 was transplanted at the time a breast chloroma was noted The chloroma disappeared following treatment with electron-beam radiotherapy (1,000 cGy), but no systemic therapy was used other than the ablative therapy described above. Patient 9 was reinduced with ARA-C (100 mg/m²/day × 7 days)/DNR (30 mg/m²/day × 3 days)/Vincristine (1 mg/m² × 1).

RESULTS

Surface marker analysis Cytofluorographic analysis of the patients' leukemia cells revealed that 21% to 91% of cells were positive for the PM-81 marker (Table 2) Complement-mediated cytotoxicity of 52–100% of cells from individual patients was achieved with this MoAb. Cells (FAB M4) from three patients (nos. 1, 8, and 10) also reacted significantly with MoAb AML-2-23 (Table 2). Cells from two patients (nos. 2 and 9) were not available for analysis since the patients were first referred to us in second remission. Our experience is that blasts from >93% of patients with FAB M4 AML react with both MoAbs PM-81 and AML-2-23.[9,11]

Engraftment. The first neutrophils appeared by posttransplant day 9 in all patients. The mean time to reach a neutrophil count of >500 cells/µL was 27 days (±5.4, range 12 to 60 days). Reticulocytes appeared in the blood on approximately day 20, and the hemoglobin levels were

Table 2. Phenotype of Patients' Leukemia Cells as Determined by Flow Cytometry and Complement-Dependent Cytotoxicity

		PM-81		AML-2-23	
		% Positive			
Patient	FAB	IF	C'	IF	C'
1	M2	77	81	32	64
2	M4	ND	ND	ND	ND
3	M2	83	90	1	15
4	M1	91	100	9	16
5	M2	67	75	3	15
6	M1	21	52	3	14
7	M1	40	55	2	16
8	M4	80	92	70	85
9	M2	ND	ND	ND	ND
10	M4	68	85	44	74

FAB, French-American-British classification; IF, immunofluorescence, ND; not done.

Blast cells from patients with AML were examined by indirect immunofluorescence and flow cytometry for the expression of the PM-81 and AML-2-23-defined antigens Background staining from irrelevant isotype control monoclonal antibodies (MoAbs) has been substracted from the percentage values shown; 10,000 cells were examined in each analysis. Cells were incubated with MoAb and rabbit complement (C') for 60 minutes, and percentage of cytotoxicity was estimated by dye exclusion (acridine orange/ethidium bromide).

sustained at >10 g/dL without transfusions of RBCs by day 39 (±5.9, range 32 to 62 days) on average. Recovery of platelets was generally slow. Although eight of the ten patients no longer required platelet transfusions by day 45 (platelet count ≥ 20,000/μL), platelet counts of >50,000/μL were not reached until a mean time of 60 ± 20 days (range 45 to 150 days). A normal platelet count was not achieved in patient 1 until at least day 280 posttransplant (Fig 1). We observed a similar time course of platelet recovery in patients 3 through 8. Two patients (nos. 2 and 7) never achieved sustained platelet counts >20,000/μL. Bone marrow biopsy in these patients showed decreased megakaryocytes and slight hypocellularity. One ultimately died of a massive subarachnoid hemorrhage (no. 2), and one (no. 7) died of respiratory failure secondary to infection.

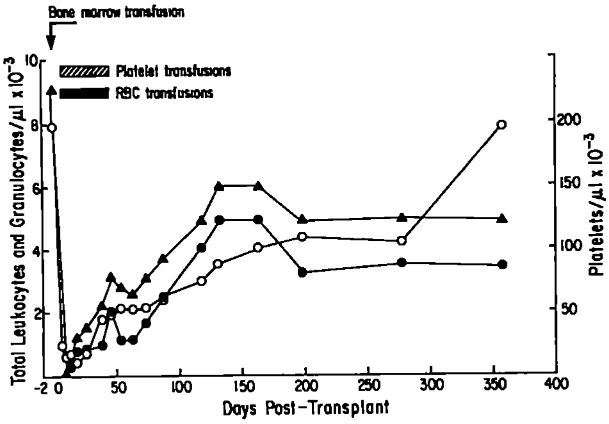

Fig 1. Blood cell counts in patient 1 following autologous bone marrow transplantation (day 0). Granulocyte (●) and total leukocyte (▲) counts are shown on the left vertical axis; platelet counts (○) are shown on the right vertical axis. The periods of time during which the patient received RBC or platelet transfusions are shown in the boxes

Toxicity. Patients suffered the expected side effects of radiotherapy and cyclophosphamide, including nausea, vomiting and mucositis. All patients required treatment with multiple antibiotics, including amphotericin B for proven or suspected candidiasis. Patients 2 and 7 had persistent thrombocytopenia which contributed to their deaths. There was no evidence of leukemia pre- or postmortem in either patient.

Effect of MoAb + C' treatment on CFU-GM. Bone marrow cells before and after MoAb + C' treatment were cultured for 14 days in methylcellulose and examined for CFU-GM content. The number and concentration of CFU-GM varied from patient to patient before MoAb + C' treatment, reflecting at the least a variation in the assay from experiment to experiment and possibly in the true number of CFU-GM harvested (Table 1). The degree of variation after MoAb + C' treatment was less significant. Overall, the effect of MoAb + C' was to decrease CFU-GM absolute numbers by an average of 45%.

Outcome Seven patients are surviving disease-free following ABMT (Table 3). The longest survivors are 630+, 450+, 380+, and 300+ days posttransplant. These patients are enjoying a high quality of life and are hematologically normal or nearly so. One patient who was in partial remission died following relapse of leukemia, and two died while still in leukemic remission 230 and 180 days posttransplant. In summary, six of seven patients transplanted in second or third CR are surviving and disease-free at 60+, 90+, 180+, 390+, 450+, and 630+ days.

DISCUSSION

We have described our initial experience in a phase I study of the use of antimyeloid MoAbs to treat remission bone marrow ex vivo prior to its autotransplantation in patients with AML. This is the first report of the use of MoAbs directed to myeloid-differentiation antigens for this purpose and is significant for two reasons. First, although engraftment of normal myeloid cells was delayed, all patients ultimately recovered normal granulocyte levels despite significant reductions in the absolute numbers of CFU-GM after MoAb treatment (45% on average). The second significant albeit preliminary observation is that long periods of leukemia-free survival have resulted from this program in patients who historically would have developed early relapses. Although the number of patients we have treated is small, the patients' remission durations are promising. The three patients transplanted in second CR with the longest follow-up are surviving at 180+, 390+, and 630+ days; another patient in second CR (no. 2) who died of thrombocytopenia was in leukemic remission at 230 days posttransplant. The expected median survival of patients in second remission following high-dose ARA-C reinduction is ≤5 months.[20] All our patients transplanted in second CR who have been followed long enough have exceeded 5 months of disease-free survival. The patient transplanted in third CR is surviving and disease-free 450+ days posttransplant This duration of remission is rare in third remission. Although these initial results are encouraging, definitive conclusions

Table 3. Summary of Clinical Data and Transplant Outcome of Patients Who Underwent ABMT

Patient	Age/Sex	Duration First CR (mo)	Duration Second CR (mo)	Remission Status at		Disease-Free Survival After ABMT (mo)
				Harvest	Transplant	
1	20/M	30	1*	Second CR	Second CR	21.0+
2	32/F	10	3*	Second CR	Second CR	7.7
3	44/F	31	10	Third CR	Third CR	15.0+
4	36/F	18	1*	First CR	Second CR	13.0+
5	43/M	13	2	Second CR	Third PR	3 3
6	28/F	20	—	First CR	First CR†	10.0+
7	16/F	10	—	First CR	First†	6 0
8	40/M	20	1*	First CR	Second CR	6.0+
9	44/M	9	1*	Second CR	Second CR	3 0+
10	44/F	12	1*	First CR	Second CR	2.0+

*Duration of second CR before autologous bone marrow transplantation (ABMT).

†Early relapse

regarding the curative potential of this program require more time, more patients, and a larger clinical trial

It will be important to determine whether these initial promising results are related to the in vitro MoAb treatment. For example, ABMT in AML without purging of leukemia cells has been reported.[21-25] When performed in relapse or second remission, ABMT has not produced long remissions[21,22]; this method has been more successful in first remission, however.[23-25] Eleven of 29 (38%) patients in first CR have achieved long disease-free survival (≥2 years) in these studies, with an overall median survival of 63 weeks. Fourteen of 29 patients in these trials relapsed within 24 months, however. In addition to immunologic methods, cytotoxic drugs are potentially useful for purging marrow of residual leukemia blasts. Kaizer and colleagues reported several long-term survivors with AML transplanted with autologous marrow treated in vitro with the cyclophosphamide congener, 4-hydroperoxycyclophosphamide (4HC).[26] Definitive clinical trials will be necessary to demonstrate the relative efficacy of these various therapeutic approaches.

This study addresses the fundamental scientific question of whether it is possible to eliminate the leukemic clone of cells from patients with AML using MoAbs directed to antigens that normally appear at the level of the CFU-GM. Thus, the therapeutic advantage we wish to exploit is based on our observation that clonogenic leukemia cells generally express the antigens we are targeting, whereas primitive normal progenitor cells do not.[14] We are attempting to determine if leukemia cells from individual patients arise from a pluripotent progenitor cell population or from a later progenitor cell commmitted to the granulocyte/monocyte lineage. From the work of Fialkow and colleagues,[27] examining clonality of various blood elements in patients with AML, one might predict that our treatment would be successful for some patients but not for patients whose cells had a more immature origin. Studies of the expression of cell surface antigens on AML cells and their progenitors have revealed that antigens are acquired in an orderly fashion.[14,28] analogous to normal myeloid differentiation. Thus, it may be possible to correlate the antigenic phenotype of patients' leukemia cells with the outcome of transplantation with MoAb-treated bone marrow in these patients. We examined the total leukemia cell population for antigen expression in the patients selected for this pilot study by flow cytometry and C'-dependent lysis. It may be more relevant to consider the phenotype of clonogenic leukemia cells. We were not able to measure the effect of MoAb therapy on clonogenic leukemia cells (L-CFC) from the patients reported in this study. We are encouraged, however, because a single exposure to MoAb PM-81 clone eliminated >50% of the L-CFC from 13 to 20 patients with AML in another study, whereas MoAb AML-2-23 eliminated >50% of L-CFC from 6 of the same 20 patients. This indicates that the antigens defined by these MoAbs are expressed at the level of the clonogenic leukemia cell in some patients with leukemia. The heterogeneity in antigen expression on L-CFC could allow some L-CFC to escape lysis, however. In a model system for clonogenic leukemia cells using the HL-60 leukemia cell line, we found that the degree of clonogenic cell kill can be increased by using two treatments rather than one[18] and by using two MoAbs together (unpublished observations, June 1985). Thus, under such optimal conditions of C'-mediated lysis, the elimination of L-CFC is possibly greater than that predicted from the study by Sabbath and co-workers.[14] Consequently, we are presently treating all bone marrows with both AML-2-23 and PM-81 whether the patients' cells react with both MoAbs or only with PM-81. The ultimate success of this program depends on our ex vivo procedure, the efficacy of the preparative regimen, and the modern support services that have contributed greatly to improved survival of leukemia patients.

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REFERENCES

1. Gale RP· Advances in the treatment of acute myelogenous leukemia. N Engl J Med 300.1189, 1979

2. Santos GW, Tutschka PJ, Brookmeyer R, Saral R, Beschorner WE, Bias WB, Braine HG, Burns WH, Elfenbein GJ, Kaizer H, Mellits D, Sensenbrenner LL, Stuart RK, Yeager AM Marrow transplantation for acute nonlymphocytic leukemia after treatment

with busulfan and cyclophosphamide. N Engl J Med 309·1347, 1983

3 Appelbaum FR, Dahlberg S, Thomas ED, Buckner CD, Cheever MA, Clift RA, Crowley J, Deeg HJ, Fefer A, Greenberg PD, Kadin M, Smith W, Stewart P, Sullivan K, Stork R, Weiden P: Bone marrow transplantation or chemotherapy after remission induction for adults with acute nonlymphoblastic leukemia. Ann Intern Med 101.581, 1984

4. O'Reilly RJ Allogeneic bone marrow transplantation: Current status and future directions. Blood 62.941, 1983

5 Ritz J, Sallan SE, Bast RC: Autologous bone marrow transplantation in CALLA-positive acute lymphoblastic leukemia after in vitro treatment with J5 monoclonal antibody and complement. Lancet 2:60, 1982

6 Ball ED, Graziano RF, Shen L, Fanger MW. Monoclonal antibodies to novel myeloid antigens reveal neutrophil heterogeneity. Proc Natl Acad Sci USA 79 5374, 1982

7. Ball ED, Graziano RF, Fanger MW. A unique antigen expressed on myeloid cells and acute leukemia blast cells defined by a monoclonal antibody. J Immunol 130:2937, 1983

8. Griffin J, Nelson D, Davis R, Davis F, McIntyre OR, Minowada J, Bloomfield C. Clinical correlations of surface antigen phenotypes in acute non-lymphocytic leukemia (ANLL): A prospective, multi-institutional study Am Soc Clin Oncol Abstr C-760:195, 1984

9. Ball ED, Fanger MW. The expression of myeloid-specific antigens on myeloid leukemia cells: Correlations with leukemia subclasses and implications for normal myeloid differentiation. Blood 61 456, 1983

10. Ball ED, Kadushin JK, Schacter B, Fanger MW: Studies on the ability of monoclonal antibodies to mediate complement-dependent lysis of human myeloid leukemia cells. J Immunol 128:1476, 1982

11. Ball ED, Fanger MW. The use of myeloid-specific monoclonal antibodies in the diagnosis of leukemia. Diagnostic Immunol 1.90, 1983

12. Magnani JL, Ball ED, Fanger MW, Hakomori S, Ginsburg V: Monoclonal antibodies PMN6, PMN29 and PM81 bind differently to glycolipids containing a sugar sequence occurring in lacto-*N*-fucopentaose III Arch Biochem Biophys 233·501, 1984

13. Maliszewski CR, Ball ED, Graziano RF, Fanger MW: Isolation and characterization of My23, a myeloid cell-derived antigen reactive with the monoclonal antibody AML-2-23. J Immunol 135:1929, 1985

14 Sabbath K, Ball ED, Larcom P, Griffin JD: Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 75:746, 1985

15. Ball ED, Bernier GM, Cornwell GG III, McIntyre OR, O'Donnell JF, Fanger MW: Monoclonal antibodies to myeloid differentiation antigens: In vivo studies of three patients with acute myeloid leukemia. Blood 62:1203, 1983

16. Lee SK, Singh J, Taylor RB: Subclasses of T-cells with different sensitivity to cytotoxic antibody in the presence of anesthetics. Eur J Immunol 5.259, 1975

17. Wiener RS, Richman CM, Yankee RA. Semicontinuous flow centrifugation for the pheresis of immunocompetent cells and stem cells. Blood 49·391, 1977

18. Howell AL, Ball ED: Monoclonal antibody-mediated cytotoxicity of myeloid leukemia cells: An in vitro model for estimating efficiency and optimal conditions for cytolysis Blood 66:649, 1985

19. Evans GE: Radiobiological considerations in magna-field irradiation Int J Radiat Oncol Biol Phys 9.1907, 1983

20 Herzig RH, Lazarus HM, Wolff SN, Phillips GL, Herzig GP: High-dose cytosine arabinoside therapy with and without anthracycline antibiotics for remission reinduction of acute nonlymphocytic leukemia. J Clin Oncol 3:992, 1985

21 Dicke YA, Spitzer G, Peters L, McCredie KB, Zander A, Verma DS, Vellekoop L, Hester J: Autologous bone marrow transplantation in relapsed adult acute leukemia. Lancet 1.514, 1979

22. Herve P, Rosenbaum A, Plouvier E, Flesch M, Cahn HY, Farradji A, Bergerat JP, Adjizian JC, Pignon B: Autologous bone marrow transplantation in acute myelogeneous leukemia in relapse or in complete remission Can Treat Rep 66:1983, 1982

23. Stewart P, Buckner CD, Bensinger W, Appelbaum F, Fefer A, Clift R, Storb R, Sanders J, Meyers J, Hill R, Thomas ED: Autologous marrow transplantation in patients with acute nonlymphocytic leukemia in first remission. Exp Hematol 13·267, 1985

24 Lowenberg B, Abels J, Van Bekkum DW, Djoljio G, Hagenbeek A, Hendriks WDH, van de Pool J, Sizoo W, Sintnicolaas K, Wagemaker G: Transplantation of non-purified autologous bone marrow in patients with AML in first remission. Cancer 54:2840, 1984

25 Burnett AK, Watkins R, Maharaj D, McKinnon S, Tansey P, Alcorn M, Singer CRJ, McDonald GA, Robertson AG Transplantation of unpurged autologous bone marrow in acute myeloid leukaemia in first remission Lancet 2:1068, 1984

26. Kaizer H, Stuart RK, Brookmeyer R, Beschorner WE, Braine HG, Burns WH, Fuller DJ, Korbling M, Mangan KF, Saral R, Sensenbrenner L, Shaddock RK, Shende AC, Tutschka PJ, Yeager AM, Zinkham WH, Colvin OM, Santos GW. Autologous bone marrow transplantation in acute leukemia. A phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide to purge tumor cells. Blood 65:1504, 1985

27. Fialkow PJ, Singer JW, Adamson JW, Vaidya K, Dow LW, Ochs J, Moohr JW· Acute nonlymphocytic leukemia: Heterogeneity of stem cell origin. Blood 57.1068, 1981

28. Lowenberg B, Bauman JGL: Further results in understanding the subpopulation structure of AML: Clonogenic cells and their progeny identified by differentiation markers. Blood 66:1225, 1985

Interleukin-1 (22-K Factor) Induces Release of Granulocyte-Macrophage Colony-Stimulating Activity From Human Mononuclear Phagocytes

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An electrophoretically pure preparation of natural human interleukin-1 (IL-1) was shown to stimulate in vitro colony formation in human bone marrow cultures. Day 4 myeloid cluster-forming cells (CFC), as well as early (day 7) and late (day 10) granulocyte-macrophage colony-forming units (CFU-GM) were stimulated in a dose-dependent fashion. At optimal concentrations of IL-1, the number of day 4 CFC reached 72%, the number of day 7 CFU-GM reached 32%, and the number of day 10 CFU-GM reached 80% of the respective numbers of colonies obtained by addition of crude leukocyte-conditioned medium (LCM). The IL-1–induced stimulatory effect on CFU-GM growth could be completely neutralized by a rabbit anti–IL-1 antiserum. Colony growth was abrogated by depleting the marrow cell suspensions of phagocytic cells prior to IL-1 addition. Conversely, the effect could be reintroduced by addition of marrow-derived adherent cells to bone marrow cell suspensions that had been depleted of both phagocytic and E rosetting T cells. Furthermore, media conditioned by bone marrow-derived adherent cells or by peripheral blood mononuclear phagocytes in the presence but not in the absence of IL-1, stimulated in vitro colony growth of phagocyte-depleted bone marrow cell suspensions. These results indicate that IL-1 induces release of granulocyte-macrophage colony-stimulating activity (GM-CSA) from human mononuclear phagocytes.

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IN VITRO GROWTH of hematopoietic colony-forming cells in semisolid media requires the continuous presence of appropriate growth factors. Proliferation of granulocyte-macrophage (GM) precursors to colonies of mature progeny occurs under the influence of specific GM colony-stimulating factors (GM-CSF), also functionally designated as GM colony stimulating-activity (GM-CSA)[1] In humans, GM-CSF has been produced by various tissues and cell types, ie, T lymphocytes,[2,3] endothelial cells,[4] marrow adherent cells,[5] and monocytes.[6-8] Factors that regulate GM-CSF production by these cells are still largely unknown, however. Recent evidence has suggested a possible role for interleukin-1 (IL-1) in the regulation of granulopoiesis. Multiple injections of partially purified rabbit leukocytic endogenous mediator (LEM), a protein generally recognized as IL-1, resulted in a sustained elevation of the level of circulating neutrophils and increased plasma levels of GM-CSF.[9]

Preliminary studies in our laboratory using electrophoretically pure preparations of a human IL-1–related factor showed in vitro stimulation of colony formation in human bone marrow cultures.[10] This factor, obtained from mitogen-stimulated leukocytes, was discovered by its antiviral effect, ie, the capacity to stimulate fibroblasts to produce interferon.[10,11] It could be identified as a human IL-1 β by its spectrum of biologic activities and its *N*-terminal amino acid sequence.[10] The profound hematological effects observed after in vivo administration of this IL-1 also indicated its possible role in granulopoiesis.[10] The present report provides evidence that IL-1 stimulates the production of GM-CSA by mononuclear phagocytes.

MATERIALS AND METHODS

Normal human bone marrow. After patients gave their informed consent, bone marrow was obtained by aspiration from the posterior iliac crest of hernia nuclei pulposi patients, who underwent laminectomy The cells were collected in Hanks' balanced salt solution with 100 U/mL of preservative-free heparin. After dilution in $RPMI_{1640}$ with 5% fetal bovine serum (FBS; GIBCO, Grand Island, NY), the cell suspension was separated over Ficoll-Isopaque (1.077 g/cm³, 1,000 g; 20 minutes, 20 °C), and the interphase was harvested and washed three times in RPMI + 5% FBS.

Bone marrow cell suspensions were depleted from mononuclear phagocytes by carbonyl iron incorporation.[12] First, 10 mL of unseparated cell suspension was diluted to 25 mL in RPMI and incubated with 125 mg of sterile carbonyl iron powder (Grade SF, Aristopharm, Delft, The Netherlands) in Erlenmeyer glass flasks (45 minutes, 37 °C, with continuous agitation) to allow phagocytosis of the iron particles by mononuclear phagocytes A sterile magnet was then added, and the cell suspension was shaken for five minutes. The cells were collected, and myeloid cells, RBCs, and residual iron-loaded mononuclear phagocytes were removed by sedimentation through Ficoll-Isopaque; the interphase was harvested T lymphocytes were then removed from the Ficoll interphase cells by being rosetted with 2-aminoethylthiouronium bromide (AET)-pretreated sheep erythrocytes and by sedimentation of the rosetted cells through Ficoll-Isopaque.[13] The nonrosetting interphase cells contained <2% T lymphocytes as determined with indirect immunofluorescence microscopy using monoclonal antibody Leu-4 (Becton Dickinson, Oxnard, CA) and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin antisera (Nordic Immunological Laboratories, Tilburg, The Netherlands), and <2% mononuclear phagocytes as determined with α-naphthylbutyrate esterase staining T lymphocytes were recovered from the pellet by lysis of the sheep erythrocytes with NH_4Cl.[14] These suspensions contained ≤1% α-naphthylbutyrate esterase–positive cells.

Mononuclear phagocytes from bone marrow Mononuclear phagocytes from bone marrow were obtained by adherence to

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plastic.[15] First, 10^7 interphase bone marrow cells in 4 mL of RPMI + 20% FBS were incubated in Petri dishes (φ 9 cm; 90 minutes, 37 °C) After the nonadherent cells were removed with the supernatant, the adherent cells were washed twice with RPMI + 5% FBS (20 °C) to remove residual nonadherent cells The adherent cells then were incubated in RPMI + 20% FBS (60 minutes, 4 °C). The detached cells were harvested, and the residual adherent cells were removed with a rubber spatula. Residual T lymphocytes were removed from the suspensions of adherent cells by being rosetted with AET-pretreated sheep erythrocytes and by sedimentation through Ficoll-Isopaque Mononuclear phagocyte suspensions contained >75% α-naphthylbutyrate esterase–positive cells, <1% Leu-4–positive cells, and up to 25% chloroacetate esterase–positive cells. Suspensions of bone marrow adherent cells that were used to condition culture medium in the presence or absence of IL-1 contained <1%, 1.5%, 8%, and 10% E rosetting T lymphocytes (Table 1).

Mononuclear phagocytes from peripheral blood. Mononuclear phagocytes from peripheral blood were isolated as reported previously.[16] In brief, mononuclear cells were obtained by centrifugation on Ficoll-Isopaque (1.077 g/cm³). After being washed, the cells were incubated in 25 mmol/L of Tris-HCl buffer (30 minutes, pH 7 4, 37° C), supplemented with 5% FBS. The cells were then washed in RPMI + 5% FBS and resuspended to a concentration of 25 to 50 × 10^6/mL in 2 mL of Percoll solution (Pharmacia Fine Chemicals, Woerden, The Netherlands; 1.063 g/cm³) at 4 °C. After centrifugation (10 minutes, 1,500 g, 4 °C), mononuclear phagocytes were collected from the interphase and washed twice in RPMI + 5% FBS Residual T lymphocytes were then removed by being rosetted with AET-pretreated sheep erythrocytes and by sedimentation through Ficoll-Isopaque. The remaining cell suspension contained 93% to 95% α-naphthylbutyrate esterase-positive cells and <1.5% Leu-4–positive cells.

IL-1 (22-K factor). Electrophoretically pure IL-1β was obtained from crude human lymphokine preparations by a four-step purification schedule as reported previously[10] The preparation used in all experiments contained 10,000 U/mL, as tested in a standard assay for lymphocyte-activating factor (LAF) or for antiviral activity It did not contain other cytokine activities tested, including IL-1α, IL-2, and CSA. It was found to be free of bacterial endotoxins, as tested by the Limulus amoebocyte lysate assay The lysate used in these studies can detect levels of endotoxin as low as 60 pg/mL. Moreover, the pyrogenic effect of IL-1 could be clearly distinguished from that of endotoxin, since the latter effect is resistent to heat treatment whereas the former is not.[17]

Rabbit anti–IL-1 antiserum Hetero antisera against electrophoretically pure IL-1 (22-K factor) were prepared in rabbits. At a dilution of 1:1,000, the antiserum could completely neutralize the biologic activity of IL-1β (100 antiviral or LAF U/mL). The antiserum was found to have no neutralizing activity against IL-1α interferons and IL-2.[18]

Preparation of IL-1–conditioned media. Adherent bone marrow mononuclear cells, mononuclear phagocytes, or E rosetting T lymphocytes isolated from peripheral blood were incubated at a concentration of 1 × 10^6/mL in α-modified Eagle's minimal essential medium (α-MEM; Flow Laboratories, Irvine, Scotland) + 20% FBS in a fully humidified atmosphere of 37 °C and 5% CO_2 in the presence or absence of IL-1 (30 U/mL). Conditioned media were prepared in the presence of polymyxin-B (Pfizer B.V , Rotterdam, The Netherlands) (12.5 μg/mL) to neutralize possible contaminating endotoxin activity.[19] After 1 to 5 days, the cell suspensions were centrifuged, and the supernatants were harvested and stored at −80 °C until use. In all experiments these conditioned media were used at a final concentration of 20%.

CFU-GM cultures Cells were cultured in a medium containing 20% FBS (Rehatuin, Kankakee, IL), 20% leukocyte-conditioned medium (LCM),[20] or IL-1 or IL-1-conditioned medium, 30% α-MEM, and 30% methylcellulose 3% in a fully humidified atmosphere of 37 °C and 5% CO_2.[21] Six replicates of 0 1 mL, each containing 1 × 10^4 bone marrow cells, were plated into the wells of microtiter plates. Mononuclear phagocyte- and T lymphocyte-depleted bone marrow cell suspensions were cultured at a concentration of 0.25 to 0.50 × 10^4 cells per well. After four days of culture, the number of clusters of 5 to 20 cells were counted to determine the number of the day 4 cluster-forming cells.[22] After seven and ten days, the number of CFU-GM colonies, defined as granulocytic, monocytic, or eosinophilic aggregates of >20 cells, were scored. Normal values in our laboratory for day 4 CFC are. 269 ± 25 (mean ± SE), for day 7 CFU-GM: 82 ± 7, and for day 10 CFU-GM: 182 ± 15/10^5 bone marrow mononuclear cells plated. Cultures to which 20% crude LCM as a source for CSF was added were included as positive controls, determining the 100% colony growth. To study the effect of IL-1 to induce colony formation, LCM was replaced by various concentrations of IL-1 diluted in α-MEM or by IL-1-conditioned medium. As a negative control, no LCM or IL-1 was added to the culture medium. Individual colonies were picked from the microtiter plates and stained with Giemsa, chloroacetate, and α-naphthylbutyrate esterase to assess eosinophilic, granulocytic, or monocytic lineage, respectively.

Table 1. Effect of Media Conditioned by Bone Marrow Adherent Cells, Peripheral Blood Mononuclear Phagocytes, or E Rosetting T Cells in the Presence or Absence of IL-1 on Day 10 CFU-GM Growth

	Percentage of Total No of Colonies Obtained in Presence of LCM					
	Culture Medium Conditioned by					
	Marrow Adherent Cells (ADH-mφ)*		Blood Mononuclear Phagocytes (mφ)†			
Incubation Time (h)	ADH-mφ	ADH-mφ + IL-1	mφ	mφ + IL-1	IL-1*†	E Rosetting T Cells + IL-1‡
24	20 ± 14	77 ± 10	0 ± 0	88 ± 1	0 ± 0	0 ± 0
48	11 ± 11	100 ± 15	0 ± 0	106 ± 11	0 ± 0	0 ± 0
72	19 ± 13	102 ± 15	0 ± 0	102 ± 11	0 ± 0	0 ± 0
120	—	—	0 ± 0	116 ± 0	0 ± 0	0 ± 0

IL-1, interleukin-1 (30 U/mL); LCM, leukocyte-conditioned medium, CFU-GM, granulocyte-macrophage colony-forming unit, ADH – mφ, marrow adherent cells; mφ, mononuclear phagocyte.

Data are expressed as mean ± SE of 4, 3, and 2 experiments. Conditioned media were tested on mononuclear phagocyte- and T-cell depleted bone marrow cells.

*Four experiments.

†Two experiments.

‡Three experiments.

For statistical analysis, differences were evaluated by the paired *t* test.

RESULTS

Stimulatory activity of IL-1 on nondepleted bone marrow cells. Serial threefold and tenfold dilutions of IL-1 were tested for their effect on in vitro colony formation in nondepleted bone marrow cultures. The results of these experiments are illustrated in Fig 1. The numbers of colonies obtained with LCM were within the normal limits of our laboratory. A dose-dependent stimulatory effect on day 4 CFC and on early (day 7) as well as late (day 10) CFU-GM was noted. At the optimal concentration of IL-1 (30 U/mL), the number of colonies was still inferior to that obtained in the presence of LCM. Also, relatively few day 7 colonies were obtained, and these colonies were almost exclusively of granulocytic lineage. The day 10 CFU-GM colonies obtained with the IL-1 preparation of 30 U/mL were for 16% ± 2% (mean ± SE) of monocytic lineage and for 8% ± 2% of eosinophilic lineage; in the presence of LCM, these percentages were 18% ± 2% and 7% ± 2%, respectively The numbers of day 14 colonies were similar to those of day 10 colonies, indicating that the maximal stimulating effect had been reached on day 10 (data not shown). From these experiments, a concentration of 30 U/mL of IL-1 was chosen as an optimal working concentration.

Antiserum neutralization of IL-1. A specific rabbit anti–IL-1 antiserum was tested for its ability to neutralize the stimulatory effect of IL-1 on in vitro colony formation. When antiserum (1:20 dilution) and IL-1 (10^2 to 10^3 U/mL) were preincubated (30 minutes, 37 °C) prior to addition to the culture medium, colony growth was completely inhibited ($P < .01$; Table 2). This effect was not due to the preincubation procedure as such, since IL-1 dependent colony growth was not reduced following preincubation of IL-1 and culture medium. Moreover, the antiserum itself did not inhibit colony growth; nor did it neutralize LCM activity, since normal colony growth was obtained after preincubation of LCM and antiserum (Table 2).

Stimulatory effect of IL-1 on phagocyte- and/or T lymphocyte-depleted bone marrow cells. To test whether accessory cells were required for IL-1-dependent colony formation, depletion studies were performed (Table 3). When bone marrow cells were depleted of mononuclear phagocytes, IL-1–dependent colony formation was abrogated ($P < .001$). The reduction was not due to a lesser viability of the bone marrow cells, since normal numbers of colonies were obtained in the depleted cultures when LCM was added (Table 3).

To define the nature of the accessory cells required for the IL-1 action, reconstruction experiments, involving addition of marrow adherent cells and/or T lymphocytes to the depleted bone marrow cultures, were done. Addition of marrow adherent cells restored IL-1–induced colony formation (Fig 2), a direct relation being present between the degree of stimulation and the numbers of adherent cells added. T lymphocytes added alone or in combination with adherent cells did not contribute to the colony formation. To exclude the possibility that this IL-1–dependent colony for-

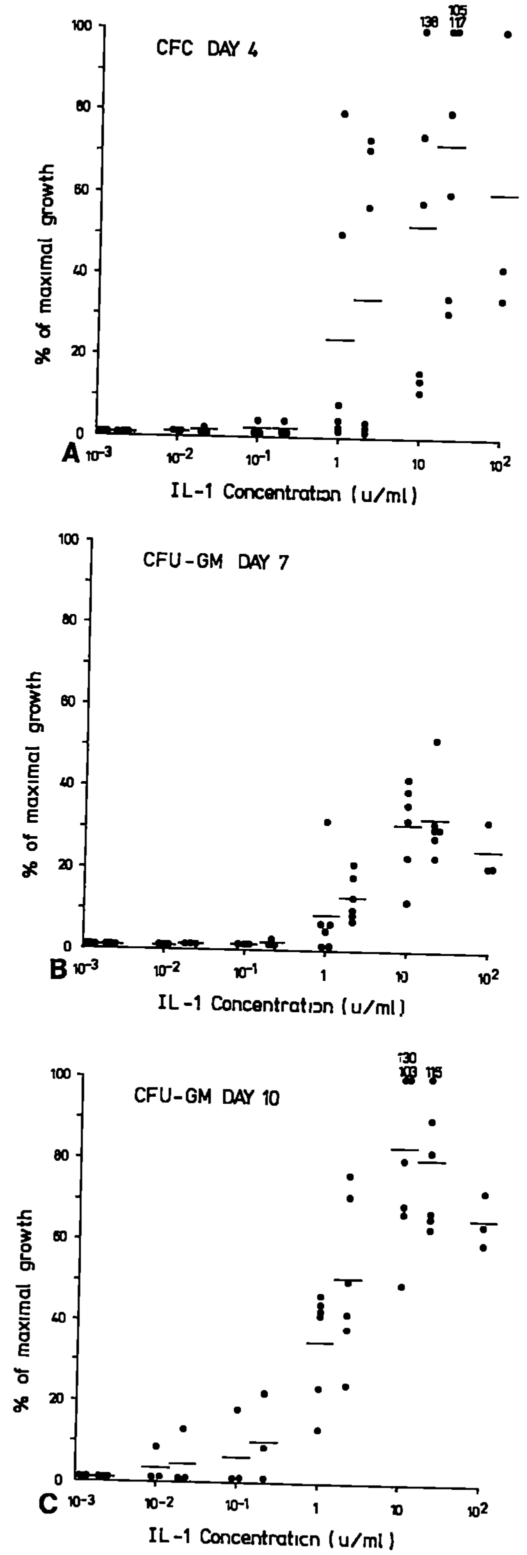

Fig 1. Effect of IL-1 on in vitro colony formation of the day 4 cluster-forming cells (CFC-4; A) and on early (day 7; B) and late (day 10; C) CFU-GM. Maximal colony growth is determined by the number of clusters or colonies per 10^5 cells obtained in the presence of 20% leukocyte-conditioned medium. IL-1 concentration is expressed as the final concentration in the culture medium. Horizontal bars: mean of three to six experiments.

Table 2. Effect of Rabbit Anti–IL-1 Antiserum on IL-1–Induced Colony Growth

Preincubation	No. of Day 10 CFU-GM Colonies as Percentage of LCM Control Exp. 1	 Exp 2
IL-1 10^3 U/mL	66	64
IL-1 10^3 U/mL + anti–IL-1 antiserum*	0	2
LCM + anti–IL-1 antiserum*	82	67

IL-1, interleukin-1 (30 U/mL), LCM, leukocyte-conditioned medium; CFU-GM, granulocyte-macrophage colony-forming unit

*Dilution 1:20

mation was due to addition of CFU-GM present in the suspensions of adherent cells, these suspensions were cultured in the presence of LCM. The day 10 CFU-GM colonies cultured from these suspensions accounted for <3% of the total number of colonies obtained after addition of adherent cells to the depleted bone marrow suspensions. Furthermore, in the absence of IL-1 or LCM, no colony growth was obtained after addition of adherent cells (data not shown).

Effect of IL-1–stimulated mononuclear phagocyte- or T lymphocyte supernatants. To assess the effect of IL-1 on the release of GM-CSA into the supernatant, culture medium was conditioned by marrow adherent cells, peripheral blood mononuclear phagocytes, or T lymphocytes, in the presence or absence of IL-1. These conditioned media were then tested on phagocyte- and T lymphocyte-depleted bone marrow cell suspensions. As indicated in Table 1, media conditioned by marrow adherent cells or peripheral blood mononuclear phagocytes in the presence of IL-1–stimulated day 10 CFU-GM colony formation. In contrast, media conditioned by these cells without IL-1 induced significantly fewer colonies ($P < .01$). Furthermore, peripheral blood T lymphocyte supernatants in the presence or absence of IL-1 had no stimulatory effect. Although small clusters could be observed on day 10, they did not develop into colonies and were dissipated after 14 to 18 days of culture. Background colony formation in cultures given conditioned media derived from unstimulated marrow adherent cells was variable. Although the origin of background activity is not clear at present, the counts were lowest in cultures receiving conditioned media from cell suspensions that contained the lowest numbers of contaminating nonadherent cells.

In an additional experiment, residual IL-1 present in conditioned medium from peripheral blood mononuclear phagocytes was neutralized by addition of anti–IL-1 antiserum immediately prior to culture. The number of day 10 CFU-GM colonies obtained with media conditioned for three days by peripheral blood mononuclear phagocytes plus IL-1 was $102/10^5$ phagocyte- and T lymphocyte-depleted bone marrow cells. In the presence of the anti–IL-1 antiserum, the number of colonies was not reduced ($158/10^5$ cells), demonstrating that the stimulatory effect of these conditioned media did not depend on the presence of IL-1.

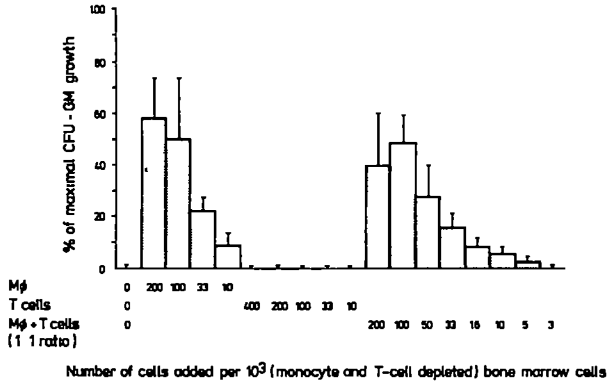

Fig 2. Effect of addition of adherent monocytes (mϕ) and T lymphocytes (T cells) to mononuclear phagocyte- and T lymphocyte-depleted bone marrow cells on IL-1 (30 U/mL) -dependent day 10 CFU-GM growth. Maximal colony growth is determined by the number of colonies per 10^5 cells obtained in the presence of 20% leukocyte-conditioned medium. Results: mean ± SE of three experiments.

DISCUSSION

The data presented in this article indicate that an electrophoretically pure IL-1 protein exerts a dose-dependent stimulatory effect on granulocytic and monocytic in vitro colony formation No IL-1–dependent stimlatory effect was observed after depletion of mononuclear phagocytic cells, whereas the effect could be reintroduced by addition of marrow adherent cells to phagocyte- and T lymphocyte-depleted bone marrow cells, suggesting that IL-1 induces the release of soluble growth factors by bone marrow adherent cells. The hypothesis that the GM-CSA was secreted by mononuclear phagocytes into the culture medium and that the presence of the mononuclear phagocytes in the CFU-GM cultures was not obligatory was confirmed by experiments in which supernatants from purified, IL-1–stimulated mononuclear phagocytes were used as conditioned medium. It is unlikely that the effect would be mediated by a potentiating

Table 3. Effect of Mϕ Depletion on IL-1–Dependent Colony Growth

	Nondepleted Marrow			mϕ-Depleted Marrow		
	Medium			Medium		
	LCM	α-MEM (%)	IL-1 (%)	LCM	α-MEM (%)	IL-1 (%)
CFC day 4	201 ± 53*	1 ± 1†	81 ± 2†	303 ± 67*	0 ± 0†	8 ± 2†
CFU-GM day 7	85 ± 17	0 ± 0	76 ± 9	96 ± 15	0 ± 0	4 ± 4
CFU-GM day 10	204 ± 38	0 ± 0	99 ± 5	167 ± 40	0 ± 0	3 ± 3

α-MEM, α-modified Eagle's minimal essential medium; CFC, cluster-forming cell; mϕ, mononuclear phagocyte; other abbreviations as in Table 1

Data are presented as mean ± SE of three experiments.

*Absolute number of clusters or colonies per 10^5 cells

†Percentage of total number of clusters or colonies obtained in the presence of LCM.

activity rather than GM-CSA, since the conditioned media were used on T lymphocyte- and mononuclear phagocyte-depleted bone marrow cells. Furthermore, because IL-1 as such had no stimulatory effect and because neutralizing concentrations of the anti–IL-1 antiserum did not inhibit the stimulatory effect of media that had been conditioned for three days by mononuclear phagocytes and IL-1, colony formation was not due to a carry-over effect of residual IL-1 on other target cell populations present in the depleted marrow cell suspensions.

In the present study, the mononuclear phagocytic lineage of the cells that released GM-CSA after stimulation by IL-1 was substantiated by the fact that media conditioned by peripheral blood mononuclear phagocytes in the presence but not in the absence of IL-1 also stimulated in vitro colony formation. The possibility that other adherent cells, eg, fibroblasts and endothelial cells, are also capable of IL-1–induced release of GM-CSA is not excluded, however. Bagby and co-workers demonstrated that GM-CSA production by these cells and by T lymphocytes is stimulated by a soluble monocyte product, termed monocyte-derived recruiting activity.[23–25] Recently, Zucali and colleagues identified the monocyte-recruiting activity that induces fibroblasts to produce GM-CSA as IL-1.[26] Therefore, IL-1 appears to be a monokine that stimulates different target cell types, ie, fibroblast and mononuclear phagocytes, to produce GM-CSA. At present, it is unknown whether other cells, ie, T lymphocytes or endothelial cells are also capable of IL-1–induced GM-CSA release.

T lymphocytes or T lymphocyte/monocyte interactions are also known to play a role in the regulation of granulopoiesis.[25,27,28] Addition of bone marrow T lymphocytes to T lymphocyte- and phagocyte-depleted marrow cells did not restore IL-1–induced colony formation, however, nor did it influence the stimulatory effect of added adherent cells. Media conditioned by peripheral blood T lymphocytes in the presence of IL-1 also lacked a stimulatory effect on colony growth. Therefore, the IL-1–induced GM-CSA release from mononuclear phagocytes appears to occur in the absence of T lymphocytes.

According to Nicola and colleagues[29] most GM-CSF from human sources contain two distinct forms. GM-CSFα preferentially stimulates day 14 colonies composed mainly of granulocytes and/or macrophages, whereas CSFβ stimulates predominantly day 7 colonies, mostly granulocytic in composition. The CSFα also stimulates eosinophilic colony formation. Both the relatively mature day 4 CFC and early (day 7), granulocytic and late (day 10) granulocytic, eosinophilic, and monocytic CFU-GM appeared to be stimulated by IL-1 in a dose-dependent fashion, indicating that both CSFα and CSFβ were released. This stimulatory effect was completely neutralized by a specific rabbit anti–IL-1 antiserum. Even at optimal doses, the stimulatory effect of IL-1 was less than that of crude LCM. This was reflected by smaller numbers of colonies and also by a smaller number of cells per colony. The proportion of mononuclear phagocytes present in the bone marrow suspension and the amount of GM-CSA produced by them are probably the factors that limit the stimulatory effect of IL-1. In reconstitution experiments, the effect of IL-1 did indeed depend on the relative number of adherent cells added, and the stimulatory effect of media conditioned by 10^6 peripheral blood mononuclear phagocytes in the presence of IL-1 was equal to that obtained with the LCM.

Because IL-1 is considered a monocyte product, our data suggest that a single mononuclear phagocyte may be both regulator and target cell. The possibility that regulator and target functions are fulfilled by different subsets of mononuclear phagocytes should also be considered, however.

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REFERENCES

1. Metcalf D. Hemopoietic colonies. In vitro cloning of normal and leukemic cells Rec Res Cancer Res 61.57, 1977

2 Parker JW, Metcalf D Production of colony-stimulating factors in mixed leucocyte cultures Immunology 26:1039, 1974

3 McNeill TA: Release of bone marrow colony stimulating activity during immunological reactions in vitro Nature (New Biol) 244·175, 1973

4 Knudtzon S, Mortensoen BT: Growth stimulation of human bone marrow cells in agar culture by vascular cells. Blood 46:937, 1975

5. Greenberg PL, Mara B, Heller P: Marrow adherent cell colony-stimulating activity production in acute myeloid leukemia Blood 52 362, 1978

6. Shah RG, Caporale LH, Moore MAS. Characterization of colony-stimulating activity produced by human monocytes and phytohemagglutinin-stimulated lymphocytes. Blood 50:811, 1977

7. Pike B, Robinson WA: Human bone marrow colony growth in agar gel. J Cell Physiol 76.77, 1970

8. Chervenick PA, Lo Buglio AF: Human blood monocytes: Stimulation of granulocyte and mononuclear colony formation in vitro. Science 178:164, 1972

9 Kampschmidt RF, Upchurch HF. Possible involvement of leukocytic endogenous mediator in granulopoiesis Proc Soc Exp Biol Med 155.89, 1977

10. Van Damme J, De Ley M, Opdenakker G, Billiau A, De Somer P, Van Beenum J: Homogeneous interferon-inducing 22K factor is related to endogenous pyrogen and interleukin-1 Nature 314:266, 1985

11. Van Damme J, Billiau A, De Ley M, De Somer P. An interferon-like or interferon-inducing protein released by mitogen-stimulated human leukocytes J Gen Virol 64:1819, 1983

12. Lundgren G, Zukoski ChF, Moller G: Differential effects of human granulocytes and lymphocytes on human fibroblasts in vitro. Clin Exp Immunol 3.817, 1968

13. Madsen M, Johnsen HE: A methodological study of E-rosette formation using AET-treated sheep red blood cells. J Immunol Methods 27:61, 1979

14 Ferrarini M, Moretta L, Abrike R, Durante ML. Receptors for IgG molecules on human lymphocytes forming spontaneous rosettes with sheep red cells Eur J Immunol 5.70, 1975

15 Messner HA, Till JE, McCulloch EA Interacting cell populations affecting granulopoietic colony formation by normal and leukemic human marrow cells. Blood 42·701, 1973

16. De Boer M, Reyneke R, Van de Griend RJ, Loos JA, Roos D.

Large-scale purification and cryopreservation of human monocytes. J Immunol Methods 43:225, 1981

17. Van Damme J, Opdenakker G, De Ley M, Heremans H, Billiau A: Pyrogenic and haematological effects of the interferon-inducing 22K factor (interleukin-1) from human leukocytes. Clin Exp Immunol 66:303, 1986

18. Billiau A, Van Damme J, Opdenakker G, Fibbe WE, Falkenburg JHF, Content J: Interleukin-1 as a cytokine inducer. Immunol Immunobiol (in press)

19. Duff GW, Atkins E: The inhibitory effect of polymyxin-B on endotoxin-induced endogenous pyrogen production. J Immunol Methods 52:333, 1982

20. Iscove NN, Senns JS, Till JE, McCulloch EA: Colony formation by normal and leukemic human marrow cells in culture: Effect of conditioned medium from human leukocytes. Blood 37:1, 1971

21. Falkenburg JHF, Jansen J, Van der Vaart-Duinkerken N, Veenhof WFJ, Blotkamp J, Goselink HM, Parlevliet J, Van Rood JJ: Polymorphic and monomorphic HLA-DR determinants on human hematopoietic progenitor cells Blood 63:1125, 1984

22. Haak HL, Goselink HM, Veenhof WFJ, Blotkamp J, Jansen J: The in-vitro growth pattern of human bone marrow in methylcellulose stimulated by different concentrations of conditioned medium. Scand J Haematol 32:515, 1984

23 Bagby GC, McCall E, Layman DL: Regulation of colony stimulating activity production. Interactions of fibroblasts, mononuclear phagocytes, and lactoferrin. J Clin Invest 71:340, 1983

24. Bagby GC, McCall E, Bergstrom KA, Burger D A monokine regulates colony-stimulating activity production by vascular endothelial cells Blood 62:663, 1983

25. Bagby GC, Rigas VD, Bennett RM, Vandenbark AA, Garewal HS: Interaction of lactoferrin, monocytes, and T-lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. J Clin Invest 68.56, 1981

26. Zucali JR, Dinarello CA, Oblon DJ, Gross MA, Anderson L, Weiner RS. Interleukin 1 stimulates fibroblasts to produce granulocyte-macrophage colony-stimulating activity and prostaglandin E_2 J Clin Invest 77:1857, 1986

27. Broxmeyer HE: Granulopoiesis, in Trubowitz S, Davis S (eds): The Human Bone Marrow, vol 1, Boca Raton, FL, CRC Press, 1982, p 145

28. Hesketh PJ, Sullivan R, Valeri CR, McCarroll LA The production of granulocyte-monocyte colony-stimulating activity by isolated human T lymphocyte subpopulations. Blood 63.1144, 1984

29. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54.614, 1979

Histocompatible Unrelated Volunteer Donors Compared With HLA Nonidentical Family Donors in Marrow Transplantation for Aplastic Anemia and Leukemia

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We treated 14 patients by transplantation of marrow from unrelated volunteer donors. Eight patients had severe aplastic anemia, 3 had chronic granulocytic leukemia, and 3 had Fanconi's anemia. The results are compared with those of a group of 14 similar patients transplanted concurrently from human leukocyte antigen (HLA)-mismatched family members: Sustained engraftment was achieved in 8 of 14 patients in both groups; one additional patient survived with autologous marrow reconstitution following an unrelated donor transplant. In the unrelated donor group, 6 of 9 evaluable patients developed grade III through IV acute graft-v-host disease, as compared with 4 of 9 patients after family-mismatched transplants. Overall survival was similar in the two groups. In the unrelated donor group 4 of 14 (29%) patients survived (median survival 1,299 days) as compared with 5 of 14 (36%) in the mismatched-family donor group (median survival 808 days). In both groups, patients with HLA phenotypically matched donors fared better than those with donors who were mismatched for one or more HLA antigen. Of the patients transplanted from HLA phenotypically matched donors 6 of 12 patients (50%) survived, as compared with 3 of 16 patients (19%) transplanted from HLA-mismatched donors. We conclude that unrelated donor bone marrow transplantation (BMT) should be considered in those cases of leukemia or bone marrow failure in which the chance of cure using conventional therapy is remote and a HLA genotypically or phenotypically matched family donor is not available.

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RECENT ADVANCES in immunobiology and transplant medicine have led to improvements in the results of human allogeneic bone marrow transplantation (BMT). Transplantation now provides the best chance of cure for many patients with hematological malignancies[1,2] and bone marrow failure.[3,4] Unfortunately, successful transplantation has largely been confined to those patients who have human leukocyte antigen (HLA) genotypically identical sibling donors. In Western Europe and North America, the average size of families restricts the number of patients with HLA-identical sibling donors to approximately one-third of the total number requiring allogeneic BMT.

Bone marrow transplantation using HLA haplotype-mismatched family donors for patients with acute leukemia has been investigated by the Royal Marsden Group.[5] Major difficulties were encountered with severe acute graft-v-host (GVHD) disease and the development of a generalized capillary leak syndrome characterized by low central venous pressure, pulmonary edema, and acute renal failure. Eleven of the 35 patients (31%) survived for >6 months after transplantation. More encouraging results are reported from Seattle, where patients with acute leukemia in remission transplanted from one HLA antigen-mismatched family donors had a projected survival similar to that of a control group transplanted from HLA-identical sibling donors.[6]

For patients with acute leukemia without HLA-identical sibling donors, other potentially curative treatments exist, including autologous transplantation of remission marrow with or without marrow purging to remove residual leukemic cells.[7] In contrast, in the treatment of chronic granulocytic leukemia (CGL), complete remission cannot be attained with chemotherapy alone, and autografting is at present only used as palliative therapy.[8] It is therefore important to investigate allogeneic BMT further in patients with CGL who lack HLA-identical sibling donors.

Severe aplastic anemia (SAA) may be successfully treated by BMT from HLA-identical sibling donors, with survivals of 70% to 80% reported.[3,4] The use of immunosuppressive therapy with antilymphocyte globulin (ALG) is more controversial, with variable response rates of 37% to 70% in different centers.[9,10] We recently identified a group of poor prognosis patients with severe aplastic anemia defined by a neutrophil count of $<0.2 \times 10^9/L$,[11] who have an actuarial survival of only 36% at 5 years after ALG therapy. This group may be considered as candidates for unrelated donor BMT when a suitable family donor is not available. Furthermore, patients with congenital aplastic anemia (Fanconi's anemia) who exhibit a defect in DNA repair[12] and progressive generalized bone marrow failure are also candidates for unrelated donor transplantation in the absence of an HLA-identical sibling donor.

Since 1981, we have carried out a pilot study using volunteer unrelated donors to transplant 14 patients with SAA, Fanconi's anemia (FA), or chronic granulocytic leukemia (CML) who did not have HLA-identical sibling donors. During the same time, an additional 14 patients from the same disease categories have been transplanted from HLA-"mismatched" family donors in whom one or more HLA alleles were matched with the recipient on the mismatched haplotype. The incidence of sustained engraftment, GVHD, infections, and survival in the two groups have been compared.

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PATIENTS AND METHODS

Patients

We treated 14 patients by allogeneic BMT from volunteer unrelated donors In all cases, a suitable family donor was not available. During the same time, 14 patients were transplanted from HLA-mismatched family donors. Relevant patient and donor details are summarized in Table 1. All patients gave informed consent for the transplant procedure according to the Hammersmith Hospital ethical guidelines.

Donor Selection

Unrelated volunteer donors

All volunteer donors were selected from the Anthony Nolan donor panel The procedure for donor selection has been described,[13] and is briefly summarized below. The Anthony Nolan panel currently contains records of 65,000 predominantly white HLA A and B antigen-typed donors, of whom ~2,000 have been DR antigen typed. An initial computer search identified HLA A and B antigen-matched donors for each potential recipient These donors were then typed for DR antigens by standard serological techniques[14] (Tables 2 and 3) Antigen matching for A, B, and DR was complete in 6 of 14 donor/recipient pairs (phenotypic identity). In eight cases, there was one HLA antigen mismatch between donor and recipient (Tables 1 and 3).

Mixed Lymphocyte Reaction

In all cases, a mixed lymphocyte reaction was carried out using a method based on that of Sengar and Terasaki.[15] The host-ν-graft and graft-ν-host activities were calculated as follows and expressed as a relative response index (RRI)

$$\text{Host-}\nu\text{-graft RRI} = \frac{R + D_1 - R + R_1}{R + X_1 - R + R_1} \times 100$$

$$\text{Graft-}\nu\text{-host RRI} = \frac{D + R_1 - D + D_1}{D + X_1 - D + D_1} \times 100,$$

where R = patient, D = potential donor, X = HLA-mismatched third-party control, and subscript one indicates irradiated cells A RRI of >35% was considered positive. Before transplantation, the sera of the recipients were tested for lymphocytotoxic antibodies with the chosen donor's cells and found to be negative.

The volunteer donors had previously consented to donate bone marrow at the time they joined the panel. Once a volunteer was considered a potential donor for a particular patient, the donation procedure and the risks involved were discussed in detail with the potential donor by a medical member of the transplant team. Each potential donor was given the opportunity to withdraw before the final written consent was obtained In all cases, the potential donor's

Table 1. Histocompatibility and Pretransplant Clinical Data

Patient No	Age/Sex (yr)	Disease Status	HLA Antigen Mismatch	MLR HVG/GVH (% RRI)	Previous Treatment
Unrelated volunteer donors (n = 14)					
CGL/35	44/M	BC	B	NE	Splenectomy BUS
CGL/36	39/M	CP	None	−3/29	Splenectomy BUS + 6TG
CGL/102	16/M	CP	None	7/−1	No splenectomy BUS
AA/38	19/F	SAA	A ? DR	11/0 4	Failed ALG
AA/44	22/M	SAA	DR	24/12	Transfused
AA/49	27/M	SAA	DR	10/19	Failed ALG × 2, HCMP
AA/50	32/F	SAA	DR	140/133	Failed ALG
AA/58	26/M	SAA	DR	11/13	Failed ALG × 2, CyA
AA/63	18/F	SAA	None	20/11	Failed ALG
AA/66	11/M	SAA	DR	NE	Failed ALG
FA/69	10/F	FA	None	38/7	Oxymetholone, transfused
FA/72	9/F	FA	None	3/17	Oxymetholone, transfused
AA/74	18/M	SAA	DR	8/7	Failed ALG × 2, HDMP
FA/83	6/F	FA	None	3/17	Oxymethalone, transfused
HLA-mismatched family donors (n = 14)					
CGL/33	22/M	AP	A	−1/3	No splenectomy BUS + 6TG
CGL/56	33/M	BC	A, B	8/4	No splenectomy BUS + 6TG
CGL/61	8/M	CP	B	0.4/5	No splenectomy BUS + 6TG
CGL/79	25/M	CP	None	4/33	No splenectomy BUS
AA/30	12/M	SAA	None	Nonreactive	Failed ALG
AA/36	33/F	SAA	A	Nonreactive	HDMP
AA/54	18/M	SAA	None	8/0.1	Prednisolone
FA/56	13/M	FA	None	−32/16	Oxymethalone, transfused
AA/57	22/M	SAA	A, B	NE	Oxymethalone, HDMP
AA/64	20/F	SAA	A, B	NE	Failed ALG × 2
FA/77	12/M	FA	None	Nonreactive	Oxymethalone, untransfused
FA/80	5/M	FA	A, B	20/14	Oxymethalone, untransfused
FA/82	10/M	FA*	B	28/2	Oxymethalone, transfused
FA/86	12/F	FA	None	4/33	Transfused

CP, Chronic phase; AP, accelerated phase, BC, blast crisis; CyA, cyclosporine; ALG, antilymphocyte globulin, HDMP, high-dose methyl prednisolone, NE, not evaluable, HVG/GVH RRI < 10%, nonreactive; BUS, Busulphan; 6TG, 6-thioguanine

*Leukemic transformation.

Table 2. Results of Transplantation

Patient No.	HLA Antigen Mismatch	Cell Dose ($\times 10^8$/kg)	Graft	Grade a-GVHD	Chronic GVHD +/0	Survival (days)
Unrelated volunteer donors (n = 14)						
CGL/35	B	0.33 (CAM)	No take	NE	NE	D, 58
CGL/36	None	2.0	Sustained	IV (CLS)	NE	D, 17
CGL/102	None	2.6 (CAM)	Sustained	0	0	A, 220
AA/38	A, ? DR	3.3	Sustained	III	+	D, 1535
AA/44	DR	2 6	Sustained	III	+	A, 1446
AA/49	DR	1 6	NE	NE	NE	D, 27
AA/50	DR	0 23 (CAM)	No take	NE	NE	{A, 1294 autologous}
AA/58	DR	3.0	Sustained	IV (CLS)	NE	D, 21
AA/63	None	3 1	Sustained	II	–	D, 326
FA/66	DR	3.8	No take	NE	NE	D, 70
FA/69	None	4.1 (CAM)	Sustained	II	+	A, 724
FA/72	None	4 0 (CAM)	Sustained	III	NE	D, 23
AA/74	DR	1 9 (CAM)	No take	NE	NE	D, 53
FA/83	None	5.7 (CAM)	Rejected day +10	I	NE	D, 57
HLA-mismatched family donors (n = 14)						
CGL/33	A	2.2	Sustained	IV (CLS)	NE	D, 52
CGL/56	A, B	2.3 (CAM)	No take	NE	NE	D, 39
CGL/61	B	2 6 (CAM)	No take	NE	NE	D, 37
CGL/79	None	4 1 (CAM)	Sustained	III	+	A, 520
AA/30	None	2.8	Sustained	II	+	A, 1808
AA/36	A	2 8	Sustained	I	0	A, 1704
AA/54	None	2 2	No take	NE	NE	D, 45
FA/56	None	4.0	Sustained	III	+	A, 1097
AA/57	A, B	4 4	Sustained	IV	NE	D, 43
AA/64	A, B	2.9	Early death	NE	NE	D, 15
FA/77	None	2 1 (CAM)	Sustained	0	0	A, 470
FA/80	A, B	11 6 (CAM)	Rejected day +30	I	NE	D, 92
FA/82	B	4 0 (CAM)	No take	NE	NE	D, 43
FA/86	None	2.6 (CAM)	Sustained	0	0	D, 120

GVHD, gravt-v-host disease, CAM, ex vivo marrow treated with Campath-1, CLS, capillary leak syndrome.

general practitioner was notified by the transplant team and kept fully informed.

HLA-Mismatched Family Donors

All donors were HLA haploidentical parents or siblings of the patient in whom one or more loci were identical with the recipient on the mismatched haplotype. Six donor/recipient pairs were HLA phenotypically identical; in four cases one HLA antigen was mismatched, and in four cases two HLA antigens were mismatched. On mixed lymphocyte reaction testing in all cases, the RRI was <35% (Table 1 and Table 3)

Methods

Pretransplant Chemoradiotherapy Protocols

Chronic granulocytic leukemia All patients received cyclophosphamide 60 mg/kg x 2, daunorubicin 60 mg/m^2, and fractionated total body irradiation (TBI) 200 x 5 or 200 x 6 cGy at a dose rate of 15 cGy/minute. Patients CGL/79 and CGL/102 received additional total lymphoid irradiation of 600 cGy Patient CGL/102 also received pretransplant infusions of rat anti-human lymphocyte monoclonal antibodies Campath-2 (anti-CD7), Campath-3 (anti-CD3),[16] and the panlymphocyte monoclonal antibody Campath-1.[17] The Campath antibodies were kindly supplied by Dr Herman Waldmann.

Severe aplastic anemia All patients received cyclophosphamide 50 mg/kg x 4. Patients AA/30, AA/36, AA/44, and AA/63 also received procarbazine 12 5 mg/kg x 3 and horse antilymphocyte globulin 1 5 vials 10 mg x 3 (Merieux, Lyons, France) Patients AA/64 and AA/74 had longstanding SAA and were treated with TBI 200 x 3 cGy to reduce the risk of graft rejection.

Fanconi's anemia All patients received cyclophosphamide 5 mg/kg x 4 and TBI 200 cGy x 3 Patient FA/86 also received pretransplant infusions of monoclonal antibodies Campath-2, Campath-3, and Campath-1

Prevention and Treatment of GVHD

Posttransplant immunosuppression All patients received regular cyclosporine (CyA) starting either on day −4 or day −1 Patients CGL/33, CGL/36, CGL/35, AA/30, AA/36, AA/38, and FA/56 received oral CyA 12.5 mg/kg twice daily for four days, followed by 6 25 mg/kg twice daily. All other patients received CyA by intravenous (IV) infusion. A loading IV dose of 5 to 8 mg/kg/day was given for four to five days followed by 2 to 4 mg/kg/day. Oral CyA was introduced when gastrointestinal function was satisfactory. CyA was continued for 4 to 6 months after BMT for CGL and 9 to 12 months after BMT for SAA and FA.

Ex vivo treatment of donor marrow In 14 cases, the marrow was transfused without ex vivo treatment, (Table 2) In the remaining 14 cases, the marrow was concentrated using an IBM 2991 cell

Table 3. Donor and Recipient HLA Types

Patient Identity	Donor HLA Type	Recipient HLA Type
Unrelated volunteer donors (n = 14)		
CGL/35	A2,24, B15, DR 4	A2,24, B51,15, DR 4
CGL/36	A28,24, B8,25, DR 3	A28,24, B8,25, DR 3
CGL/102	A2,3 B7,15, DR 8,9	A2,3 B7,15, DR 8,9
AA/38	A2,24 B40,44, DR 7	A2,23, B40,44, DR?
AA/44	A2,29, B13,44, DR 7	A2,29, B13,44, DR 2,7
AA/49	A1,3, B8,44, DR 3,5	A1,3, B8,44, DR 3
AA/50	A2,32, B7,40, DR 2,4	A2,32, B7,40, DR 2,6
AA/58	A2,3 B5,7, DR 4	A2,3, B51,7, DR 4,7
AA/63	A1,11, B44,8, DR 2,3	A1,11, B44,8 DR 2,3
AA/66	A2,29, B12,40, DR 2,6	A2,29, B12,40, DR 2
FA/69	A3,10, B7,12, DR 2,7	A3,10, B7,12, DR 2,7
FA/72	A1,3, B7,8, DR 2,7	A1,3, B7,8, DR 2,7
AA/74	A2,3, B15,8, DR 3,6	A2,3, B15,8, DR 3
FA/83	A1,3, B7,8, DR 2,7	A1,3, B7,8, DR 2,7
HLA-mismatched family donors (n = 14)		
CGL/33	A1,—, B8,40, DR 3,4	A1,2, B8,40, DR 3,4
CGL/56	A2,28, B7,W60, DR 2,6	A2,W32, B44,W60, DR 2,6
CGL/61	A2,24, B15,—, DR 4	A2,24, B15,51, DR 4
CGL/79	A1,24, B8,14, DR 3	A1,24, B8,14, DR 3
AA/30	A1,2, B8,15, DR 3,4	A1,2, B8,15, DR 3,4
AA/36	A2,2, B7,14, DR 2,7	A2,10, B7,14, DR 2,7
AA/54	A1,2, B7,27, DR 5	A1,2, B7,27, DR 5
FA/56	Known phenotypic match, data not available	
AA/57	A1,11,—, B35,37, DR 4,7	A11,32, B14,35, DR 4,7
AA/64	A11,32, B—,55, DR 4	A2,32, B23,51, DR 4
FA/77	A1,2, B8,27, DR 3,4	A1,2, B8,27, DR 3,4
FA/80	A—,11, B—,35, DR 4	A1,11, B18, DR 4
FA/82	A2, B44,15, DR 1,4	A2, B44,40, DR 1,4
FA/86	A1,3, B7,8, DR 2,3	A1,3, B7,8, DR 2,3

washer. In 12 cases, a marrow buffy coat preparation was treated with the monoclonal antibody Campath-1[17] in a final concentration of 100 μg/mL. In cases CGL/35 and AA/50, a marrow mononuclear cell suspension prepared by Ficoll separation was treated. Although the total cell numbers in these cases were low, the final marrow *mononuclear* cell dose was comparable with that in the 26 cases in which marrow buffy coat was processed (Table 2). In all cases, 20% to 25% donor serum was used as a source of complement. Ex-vivo marrow treatment was carried out in 7 of 14 unrelated and 7 of 14 family cases (Table 2). After treatment, the marrow contained 0 to 0.5×10^7 T lymphocytes detected by a standard E rosette technique (data not shown).

Treatment of GVHD. Clinically apparent acute GVHD was graded according to the Seattle criteria.[18] In most cases, a skin biopsy was performed to confirm the diagnosis. Chronic GVHD was defined as GVHD persisting or occurring de novo >100 days after transplantation. Patients developing grade II through IV acute GVHD were treated with high-dose methyl prednisolone 10 to 20 mg/kg/day given by IV bolus injection

Protective Isolation and Antibiotic Regimens

All patients were nursed in single rooms equipped with a positive-pressure clean-air supply. Laminar airflow units were not used. Oral nonabsorbable antibiotics and IV antibiotic regimens were given as previously described.[4]

Statistical Analysis of Data

Survival curves were calculated using a nonparametric estimation for incomplete observations.[19] Survival of patients was analyzed on April 7, 1986.

RESULTS

The results of transplantation in 14 patients with unrelated donors and 14 patients with mismatched family donors are summarized in Table 2.

Survival

The survival in both groups was similar, with 4 of 14 (29%) of patients in the unrelated donor group alive and 5 of 14 (36%) of patients in the mismatched family donor group alive at the time of analysis. Median survival for unrelated donor transplants was 1,299 days (range 220 to 1,669) as compared with 808 days (range 470 to 1808) for mismatched family transplants. In both groups, survival was better following phenotypically matched transplants than after one or more antigen-mismatched transplants (Tables 2 and 4). Actuarial survival of FA and SAA patients transplanted from unrelated and family-mismatched donors as compared with similar patients with the same diagnosis transplanted concurrently from HLA-identical sibling donors is shown in Fig 1. There was no significant difference in actuarial survival between unrelated and mismatched family transplants; however, both groups survived less well than the patients transplanted from HLA-identical sibling donors. The HLA-identical sibling transplants were not directly comparable, however, as there was a much higher proportion of patients with acquired aplastic anemia in the group (47 of 50) than in the HLA-nonidentical transplant group (13 of 21). Of the 7 patients with CGL transplanted with marrow from matched unrelated or mismatched family donors, 4 were transplanted in chronic phase, and 3 were transplanted in more advanced phases of the disease (Table 2) Only two (29%) survived, both transplanted in the chronic phase. One survivor (CGL/79) recieved marrow from a phenotypically matched parent, and the other (CGL/102) received marrow from a phenotypically matched unrelated donor. These results may be compared with results of transplant using

Table 4. Survival Related to HLA Matching and Donor Category

Donor Category	Campath* (+/−)	Sustained Graft	No With III–IV GVHD/ No Evaluable	Survival
HLA MM family	4+	0	NE	3/15 (19%)
	4−	3	2/3	
HLA MM unrelated	3+	0†	NE	
	5−	3	3/3	
HLA pheno family	3+	3	1/3	7/12 (50%)
	3−	2	1/2	
HLA pheno unrelated	4+	3	2/3	
	2−	2	1/2	

Numbers correspond to number of cases in each category NE, not evaluable; MM, mismatch, pheno, phenotypic.

*Ex vivo T cell depletion with Campath-I

†One patient survives with autologous marrow reconstitution

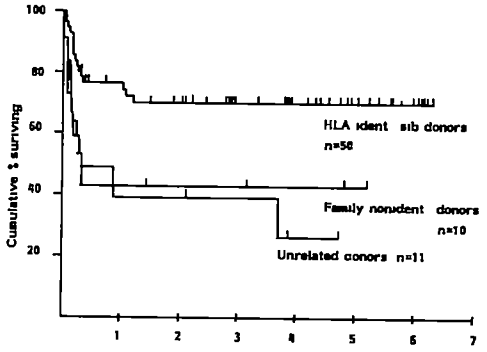

Fig 1. Acturarial survival for patients with severe aplastic anemia (SAA) and Franconi's anemia (FA) transplanted from family-mismatched or unrelated donors as compared with 50 patients with SAA and FA transplanted from human leukocyte antigen (HLA)-identical sibling donors. Vertical marks indicate surviving patients.

HLA-identical sibling donors; the actuarial survival at 2 years is 72% for patients transplanted in chronic phase and 18% for those transplanted in advanced phases of CGL.[2]

Evaluation of Engraftment

HLA Phenotypically Matched BMT

Satisfactorily sustained engraftment occurred in 10 of 12 patients receiving phenotypically identical transplants in 5 of 6 cases from unrelated donors and in 5 of 6 cases from family donors (Table 4). Transplantation with lymphocyte-depleted marrow was not obviously associated with increased graft failure in phenotypically matched transplants, although the numbers are too small for statistical evaluation Of the two graft failures, patient FA/83 rejected her graft 10 days after transplantation from a female unrelated donor Rejection was associated with the appearance of large granular reactive lymphocytes of predominantly CD8 phenotype in the peripheral blood. The other phenotypic graft failure was complete failure of graft taken in a patient with SAA (AA/54) who received marrow from his father.

HLA-Mismatched BMT

In contrast to the phenotypically identical transplants, the incidence of graft failure was high in unrelated or family transplants in which the donor was mismatched by one or more antigens and in which the marrow had been T cell depleted. Of the T cell-depleted mismatched transplants, 0 of 7 achieved stable engraftment, of whom 3 had unrelated and 4 had family donors. In mismatched transplants in which unmodified marrow was infused, sustained engraftment occurred in 6 of 7 evaluable cases.

Evaluation of GVHD

Of the 18 patients who were evaluable for acute GVHD, 10 developed grade III through IV disease. Six of these were transplanted from unrelated donors, and four were transplanted from family donors (Tables 2 and 4). An additional five patients developed grade I through II acute GVHD, and only 3 of 18 evaluable patients remained free of acute GVHD. The number of patients is too small to assess whether there is a difference in severity of acute GVHD between patients receiving marrow from phenotypically matched donors as compared with those receiving marrow from donors mismatched for one or more antigen. Three patients with grade II through IV acute GVHD developed a generalized capillary leak syndrome with pulmonary edema and acute renal failure. Two patients with capillary leak were transplanted from unrelated donors, and one was transplanted from a family HLA A antigen-mismatched donor Ten patients were evaluable for chronic GVHD, which was present in eight cases. Severe progressive chronic GVHD developed in only one patient who was transplanted from an unrelated donor with a 1 DR antigen mismatch (AA/44, Table 2).

Causes of Death

The causes of death in patients transplanted from unrelated donors and family-mismatched donors are shown in Table 5. In both groups, the commonest cause of death was bacterial or fungal infection in association with graft failure. In 7 of 9 patients dying with graft failure, the marrow donor was mismatched for one or more HLA antigens. Five patients died from acute GVHD disease and infections. In 3 of 5 of these cases, the immediate cause of death was an acute capillary leak syndrome (Table 2). There were two late deaths; one from gram-negative septicemia (day +326), and one sudden death on day +1,535 in a patient with chronic GVHD. Two patients died of hemorrhage (day +15, day +27).

DISCUSSION

Successful marrow transplantation for leukemia from an unrelated donor was first reported by the Seattle group in 1980.[20] More recently, preliminary results of transplantation from partially HLA-matched unrelated volunteer donors have been reported from Iowa.[21] In 1982, we reported the first two successful transplants for SAA using volunteer donors from the Anthony Nolan panel.[13] These two patients are both included in the present report (AA/38, AA/44).

We were discouraged from using full haplotype-mismatched family donors by the high incidence of transplant complications reported by the Royal Marsden Hospital using HLA haplotype-mismatched family donors to transplant patients for AML in first or second remission.[5] Therefore, we

Table 5. Principal Causes of Death Following BMT

Death (Total No /Cause)	Donor Category	
	Unrelated (n – 14)	Family-mismatched (n – 14)
No. of deaths (total)	10	9
Graft failure (±infection)	4 CGL/35, FA/83, AA/54, AA/66	5 AA/54, CGL/56, CGL/61, FA/80, FA/82
GVHD (±infection)	4 CGL/36, FA/72, AA/58, AA/44	2 CGL/33, AA/57
Infection	1 AA/63	1 FA/86
Hemorrhage	1 AA/49	1 AA/64

BMT, bone marrow transplantation.

have continued our study of HLA-matched unrelated volunteer donors and have carried out unrelated donor transplants in 14 patients with SAA, FA, and CGL (Table 1). A concurrent group of 14 similar patients transplanted from haplotype-mismatched family donors who were matched at one or more loci on the mismatched haplotype has been compared with the unrelated donor group (Tables 1 and 2). Although the patients are a mixed group, the following preliminary observations may be made.

The survival of patients after HLA *phenotypically* identical donor transplants both in the unrelated and family donor groups is superior to that of patients transplanted from one or more HLA antigen-mismatched donors (Table 4). In contrast, the MLR expressed as the RRI was not helpful in predicting GVHD, graft failure, or survival after unrelated or mismatched family donor transplants (Tables 1 and 2).

The number of patients is too small and the group too diverse for multivariate statistical analysis of other factors that might affect survival. Table 2, however, shows that the HLA phenotypically matched group and the HLA-mismatched group have a similar range of important variables such as diagnosis, age, and conditioning protocols. In the phenotypically identical transplants, we did not experience a high incidence of graft failure when ex-vivo T cell depletion of donor marrow was carried out (Tables 2 and 4). In contrast, in our experience of transplants in which one or more HLA antigen was mismatched, ex vivo T cell depletion of donor marrow was associated with an unacceptably high incidence of fatal graft failure regardless of the pretransplant conditioning protocol used, both in family-mismatched and unrelated donor transplants. Preclinical studies show no evidence that Campath-1 has a direct toxic effect on hemopoetic progenitor cells in the concentration used in the ex vivo T cell depletion procedure.[17] It is more probable that T cell depletion enhances graft failure by removal of donor T cells, which are cytotoxic toward residual recipient immunocompetent lymphocytes with antidonor activity. This effect may be enhanced when the donor and recipient are HLA mismatched. In those mismatched transplants in which ex vivo T cell depletion was not used, engraftment was usually satisfactory, but there was a high incidence of fatal GVHD (Tables 2 and 4).

At present, the process for identifying a well-matched unrelated donor is both costly and time-consuming. Using the Anthony Nolan panel, the most time-consuming procedure is that of DR-typing donors who have been matched for HLA A and B antigens with the patient by computer. The median time taken to select an unrelated donor was 4 months, with a range of 2 to 6 months. Although a delay of several months is acceptable for some patients, for example, those with stable chronic-phase CGL, other patients such as those with SAA may develop life-threatening complications during the "workup" period Another difficulty has been the failure to find well-matched unrelated donors for patients whose ethnic background differs from that of the majority of donors on the panel.

Our preliminary results reported here using partially HLA-matched family donors are similar to our results using unrelated volunteer donors (Tables 2 and 4). It is important to emphasize that, as with the unrelated donor transplants, the results of transplantation from phenotypically matched family donors were superior to those using one or more HLA antigen-mismatched donors. Although the numbers are small, our results using mismatched family donors do not support the recent report from the Seattle group in which the projected survival after one HLA-antigen–mismatched transplants from family donors was as good as for a control group of patients transplanted from HLA-identical sibling donors.[6] An advantage of using HLA partially matched family donors rather than volunteer donors is that the transplant is less likely to be delayed by a prolonged search for a suitable donor. A recent estimate suggests, however, that only one in ten patients evaluated for BMT in the United States had a haploidentical family member with a single allele difference on the mismatched haplotype.[6] These data show that one HLA-antigen–mismatched family donors only slightly increase the size of the family donor pool provided by HLA-identical siblings. These preliminary findings support the view that the identification of a HLA phenotypically matched donor is important for the successful outcome of transplantation in the absence of an HLA genotypically identical sibling. We therefore intend to investigate further the use of HLA phenotypically matched unrelated donors for transplantation of patients with FA, CGL, and some patients with SAA. Furthermore, our results show that the incidence of graft failure is unacceptably high in HLA one or more antigen-mismatched transplants in which ex vivo T cell depletion of donor marrow is used as GVHD prophylaxis

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REFERENCES

1 Thomas ED· Marrow transplantation for malignant disease. J Clin Oncol 1.517, 1983

2. Goldman JM, Apperley J, Jones L, Marcus RE, Goolden AWG, Batchelor JR, Reid CD, Hows JM, Gordon-Smith EC, Catovsky D, Galton DAG Bone marrow transplantation for patients with chronic myeloid leukemia N Engl J Med 314; 202, 1986

3. Storb R, Prentice RL, Thomas ED: Factors associated with graft rejection after HLA identical marrow transplantation for aplastic anaemia. Br J Haematol 55:573, 1983

4. Hows JM, Palmer S, Gordon-Smith EC: The use of cyclosporin A in allogeneic bone marrow transplantation for severe aplastic anemia. Transplantation 33·382, 1982

5 Powles RL, Morgenstern GR, Kay HEM, McElwain TJ, Clink HM, Dady PJ, Barrett A, Jameson B, Depledge MH, Watson JG, Sloane J, Leigh M, Lumley J, Hedley D, Lawler SD, Filshire J, Robinson B· Mismatched family donors for bone marrow transplantation as treatment for acute leukaemia. Lancet 1:612, 1983

6. Beatty PG, Clift RA, Mickleson EM, Nisperos BB, Flournoy

N, Martin PJ, Sanders JE, Stewart P, Buckner CD, Storb R, Thomas ED, Hansen JA: Marrow transplantation from related donors other than HLA identical siblings N Engl J Med 313:765, 1985

7 Gorin NC, Aegerter P, Parker Y. Autologous bone marrow transplantation for acute leukaemia in remission. Second European survey. Exp Hematol 13:18, 1985 (suppl 17)

8 Haines ME, Goldman JM, Worsley AM, McCarthy DM, Wyatt SE, Dowding C, Kearney L, Th'ng KH, Wareham NJ, Pollock A, Galvin MC, Samson D, Gleary CG, Catovsky D, Galton DAG. Chemotherapy and autogrouping for chronic granulocytic leukaemia in transformation: Probable prolongation of survival in some patients Br J Haematol 58·711, 1984

9. Fairhead SM, Chipping PM, Gordon-Smith EC: Treatment of aplastic anaemia with antilymphocyte globulin Br J Hematol 55.7, 1983

10 Speck B, Gratwohl A, Nissen C, Leibundgut U, Ruggero D, Osterwalder B, Burri HP, Cornu P, Jeannet M: Treatment of severe aplastic anaemia with antilymphocyte globulin or bone marrow transplantation. Br Med J 282·860, 1981

11. Marsh J, Bryett KA, Al-Hashimi S, Hows JM, Gordon-Smith EC: Treatment of aplastic anaemia with antilymphocyte globulin in the UK. Bone Marrow Transplantation 1 26, 1986 (suppl 1)

12. Sasaki MS: Is Fanconi's anaemia defective in a process essential to the repair of DNA crosslinks? Nature 257:501, 1975

13. Gordon-Smith EC, Fairhead SM, Chipping PM, Hows JM, James DCO, Dodi A, Batchelor JR Bone marrow transplantation for severe aplastic anaemia using histocompatible unrelated volunteer donors Br Med J 285:835, 1982

14. Wooley P, Griffen J, Panayi GS, Batchelor JR, Welsh K, Gibson TJ: HLA-DR antigens and toxic reactions to sodium aurothiomalate and D-penicillamine in patients with rheumatoid arthritis. N Engl J Med 303:300, 1980

15 Sengar DOS, Terasaki PI: A semimicro mixed leukocyte culture test. Transplantation 11·260, 1971

16. Waldmann H, Hale G, Cobbold S, Clark M, Tighe J, Bindon C: Rat monoclonal antibodies for bone marrow transplantation (BMT). The Campath series Exp Hematol 13:431, 1985

17 Hale G, Bright S, Chumbley G, Hoang T, Metcalf D, Munro AJ, Waldmann H: Removal of T cells from bone marrow for transplantation. A monoclonal antilymphocyte antibody that fixes human complement Blood 62:873, 1983

18. Glucksberg J, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED: Clinical manifestations of graft versus host disease in human recipients of marrow from HLA matched sibling donors. Transplantation 18:295, 1974

19 Kaplan EL, Meier P· Non-parametric estimation for incomplete observations. J Am Stat Assoc 53:457, 1958

20. Hansen JA, Clift RA, Thomas ED, Buckner CD, Storb R, Giblett ER· Transplantation of marrow from an unrelated donor to a patient with acute leukemia. N Engl J Med 303:565, 1980

21. Gingrich RD, Howe CWS, Ginder GD, Goeken NE, Fyfe MA: The partially mismatched unrelated marrow donor is an acceptable and ready resource for allogeneic marrow transplantation. Blood 64 214a, 1984 (suppl 1)

Morphological Abnormalities in the Lymphocytes of Patients With the Wiskott-Aldrich Syndrome

By Dianne Kenney, Lloyd Cairns, Eileen Remold-O'Donnell, Jeffrey Peterson, Fred S. Rosen, and Robertson Parkman

Lymphocytes from 18 patients with the Wiskott-Aldrich Syndrome (WAS) were examined by scanning electron microscopy (SEM). Most peripheral blood lymphocytes from normal individuals are covered with slender microvillus projections, but a large proportion of lymphocytes from WAS patients were found to be relatively devoid of microvilli. A lymphocyte morphology scoring system was developed to quantify the density of microvilli: Grade 4 classified those lymphocytes with >75% of the surface covered with microvilli with progressive decrements to grade 1, which were those without microvilli. The mean lymphocyte morphology score of eight normal individuals was 3.62 ± .22. The mean lymphocyte score of WAS patients was substantially lower (2.89 ± .27, *P* < .001). In addition, WAS lymphocytes often were qualitatively abnormal, with short, blunted microvilli. These morphological criteria were used to diagnose WAS from the cord blood lymphocytes of one "at-risk" patient. Thus, WAS is the first primary immunodeficiency in which morphological abnormalities have been identified that can aid in diagnosis.

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THE WISKOTT-ALDRICH syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia with platelets of reduced size and function, progressive deterioration of T lymphocyte function, an inability to produce antibodies to carbohydrate antigens, and eczema.[1-3] It is difficult to make a definitive clinical diagnosis in some patients because of variability in the immunologic manifestations of the disease.

We previously reported that WAS is characterized by a molecular defect in a lymphocyte membrane surface component, a sialoglycoprotein, gpL115.[4,5] To determine if lymphocyte abnormalities could be detected at the morphological level, lymphocytes from 18 WAS patients were examined by scanning electron microscopy (SEM) and their morphology was compared with that of lymphocytes from eight normal individuals.

METHODS

Patients. The diagnosis of WAS was based on male sex, severe thrombocytopenia with platelets of reduced size and function and, in some patients, a family history of the disorder. Many of the patients had eczema and clinical immunodeficiency. Six patients had been splenectomized; 16 had platelet counts of < 100,000/μL. Their median age was 4 years (range one day to 25 years; five patients, ≤1 year).

Cell isolation. Peripheral blood was collected with parental consent in preservative-free heparin (100 U/mL) or acid-citrate-dextrose (ACD; NIH, formula A) (0.17 mL/mL). Blood specimens were maintained at room temperature and either processed immediately or the day after shipment. All patient specimens were processed in parallel with blood from a normal individual. Mononuclear cells were isolated on Ficoll-Hypaque gradients as described,[5] plated in RPMI 1640 with 2% fetal calf serum (FCS) for one hour at 37 °C; the lymphocytes (nonadherent cells) were collected, washed, and resuspended in RPMI without serum. Lymphocytes from 25 mL of cord blood anticoagulated with ACD were isolated within three hours of birth by the same procedure. Normal thymocytes were obtained by teasing thymic tissue removed as a routine part of open heart surgery. Thymocytes were resuspended in RPMI with 2% FCS and isolated, as were blood lymphocytes.

Scanning electron microscopy (SEM). Lymphocytes, 5×10^6 (or thymocytes) in 5 mL were incubated at 37 °C for 1 hour, added dropwise to 50 mL of 1.25% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.2, and incubated for one to two hours at 37 °C. Patient and paired normal samples were processed for SEM in parallel. Fixed cells were collected by gentle suction on Millipore (0.5 μm) or polycarbonate (0.6 μm) filters, and postfixed with 1% osmium tetroxide in PBS for one hour. Samples were dehydrated with graded ethanols, critically point-dried from carbon dioxide, coated with gold/palladium (60:40) to 100 to 150 Å, examined on a scanning electron microscope (AmRay 12,000 B or JEOL 35C), and photographed at 4,000 to 7,000× magnification. All photographs were generated by a "blind" observer who was unaware of the origin of the lymphocytes.

Morphology scoring. Fifty to 200 lymphocytes from each individual in at least 15 randomly selected fields were scored independently by two observers. To aid in the evaluation of normal and WAS lymphocytes, a scoring system (summarized in Table 1 and in the Results section) was established. When morphology was intermediate, the higher score was given. One of the observers scored lymphocytes from 12 of 19 patients and 6 of 8 normal subjects in a blind fashion, (ie, without knowledge of their origin). The mean difference in the scores of the two observers for all patient and normal lymphocytes was minimal (0.17 ± 0.11). Scores reported are the mean of the two observers' scores. Scores were not influenced by choice of anticoagulant or whether lymphocytes were isolated immediately or after overnight shipment. Statistical significance was determined by the nonparametric Wilcoxan rank-sum test comparison of two groups in independent samples, and results are expressed as the mean ± SD.

RESULTS

Morphology. Preliminary examination of lymphocytes from two WAS patients revealed an apparent decrease in the frequency of cells densely covered with microvillus surface projections. To evaluate the morphological alterations of the

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Table 1. Lymphocyte Morphology Scoring

Grade	Surface Morphology	Normal Lymphocytes (%)	WAS Lymphocytes (%)
4	Villus projections on >75% of cell surface	65.8 ± 18.9	22.5 ± 13.0, $P = .001$
3	Villus projections on <75% of cell surface	29.4 ± 21.0	54.1 ± 15.3, $P = .005$
2	Ridgelike projections	2.8 ± 4.1	15.7 ± 7.9, $P = .001$
1	No projections	1.9 ± 2.5	7.7 ± 7.9, NS

Scoring system to evaluate lymphocyte morphology was based on the quality and density of surface projections found in scanning electron micrographs of peripheral blood lymphocytes of eight normal individuals and 18 WAS patients.

WAS lymphocytes and to compare them with normal lymphocyte morphology, a grading system was established based on the quality and density of surface projections on normal lymphocytes (Table 1). Lymphocytes with microvillus projections on >75% of the surface were scored as grade 4, whereas lymphocytes with microvillus projections on <75% of the cell surface were scored as grade 3 (Fig 1A). Lymphocytes with only ruffled or ridge-like projections (thymocyte-like) were scored as grade 2, whereas cells with no projections were scored as grade 1 (Figs 1B, C, and D). Normal peripheral blood lymphocytes are primarily grade 4 (65.8% ± 18.9%) and grade 3 (29.4% ± 21.0%) cells with occasional grade 2 (2.8% ± 4.1%) or grade 1 (1.9% ± 2.5%) cells (Table 1). The mean score of lymphocytes from normal individuals was 3.62 ± 0.22 ($n = 8$). Normal thymocytes were composed of >95% grade 2 cells with occasional grade 3 or grade 1 cells and had a mean score of 1.99 ± 0.01 ($n = 2$) (Figs 1B and 2). Peripheral blood lymphocytes from 18 WAS patients were analyzed; the mean score was 2.89 ± 0.27 ($n = 18$, $P < .001$ as compared with normal lymphocytes). The lower score was due to a lower percentage of grade 4 cells and a higher percentage of grade 3 and grade 2 cells (Table 1). The change in the percentage of grade 1 cells was not significant. There were no differences between the absolute lymphocyte counts of the patients and the normal individuals. The lymphocyte score of 17 of 18 patients did not overlap with the normal range (Fig 2). No change in the morphology of WAS (or normal) lymphocytes resulted from holding whole blood overnight prior to isolating lymphocytes; for example, the mean morphology scores of a single patient's lymphocytes isolated immediately and, on a separate occasion, after 24-hour shipment of whole blood, were 3.03 and 3.11, respectively. The extent of morphological alterations

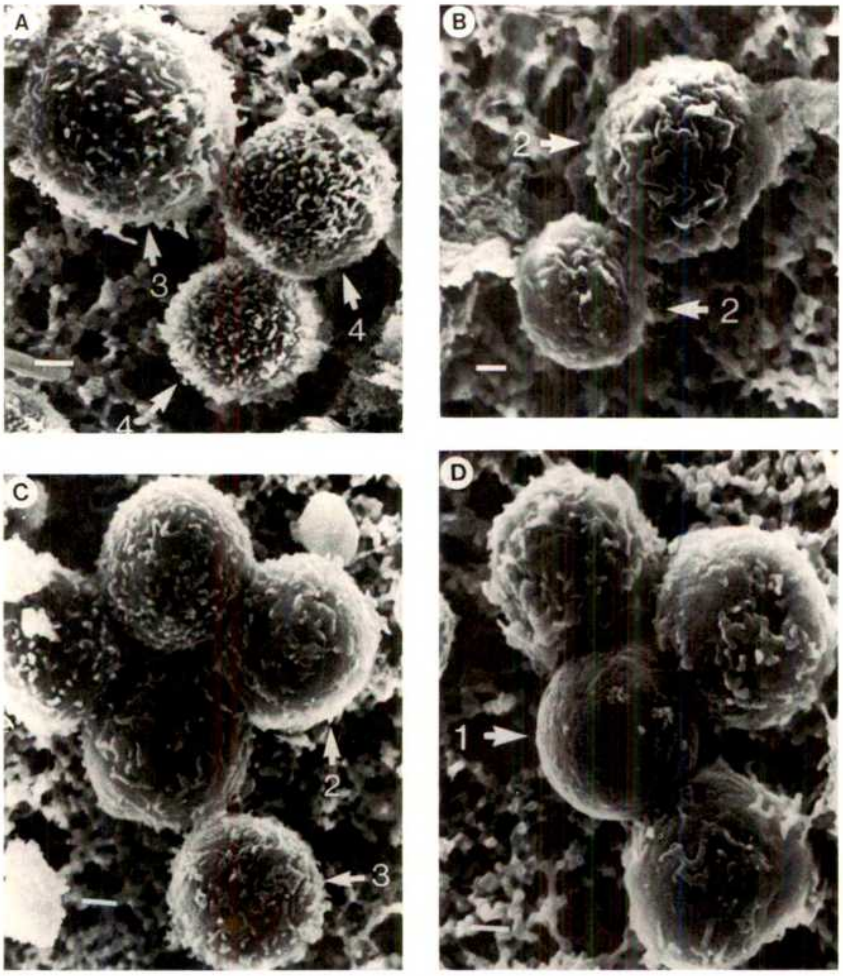

Fig 1. (A) Normal lymphocytes, grades 4 and 3; (B) normal thymocytes, grade 2; (C) WAS lymphocytes, grades 3 and 2; (D) WAS lymphocytes, grade 1. Bars = 1 μm.

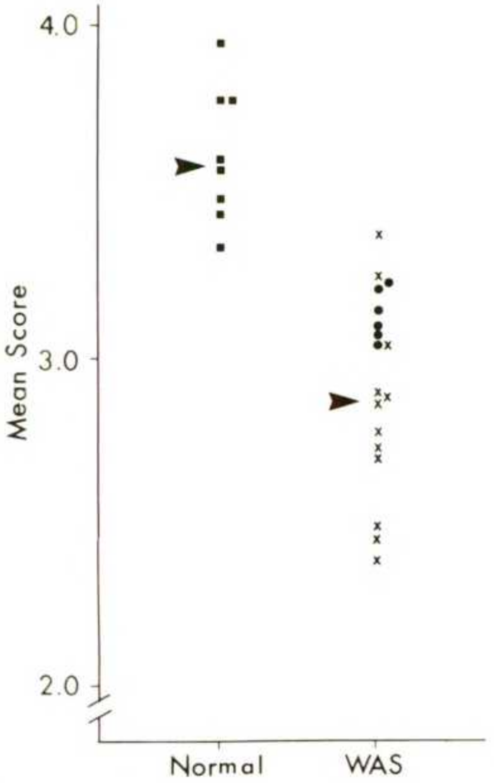

Fig 2. Mean lymphocyte morphology scores of peripheral blood lymphocytes from eight normal individuals (PBL), and 18 WAS patients (WAS). Eusplenic (x) and splenectomized (o) WAS patients are indicated. Arrows indicate mean of the scores for the normal and patient groups: PBL = 3.62 ± .22; WAS = 2.89 ± .27.

and lower numerical scores in WAS lymphocytes did not correlate with the severity of the patient's thrombocytopenia or clinical immunodeficiency (not shown). The mean morphology score of lymphocytes from the six splenectomized patients was 3.16 ± 0.13, whereas the mean score of the 12 eusplenic patients was lower—2.81 ± 0.3. Although the numerical difference between the mean scores of the two patient subgroups was not statistically significant, scores of all splenectomized patients are clustered above 3, whereas scores of eusplenic patients ranged broadly from 2.46 to 3.37 (Fig 2).

In addition to the quantitative difference reflected by the lymphocyte morphology score, qualitative differences were also noted between normal and WAS lymphocytes. The microvillus projections of many WAS lymphocytes were shorter and blunted relative to the more filamentous projections characteristic of normal peripheral blood lymphocytes (Figs 1C and D). WAS lymphocyte surface morphology was clearly different from the ruffled or ridgelike projections evident on almost all normal thymocytes, however, (compare Figs 1B and C).

Neonatal diagnosis. The neonatal diagnosis of WAS can be difficult to make even if thrombocytopenia is present; T lymphocyte function is usually normal and eczema is never present. Adequate cell numbers for surface radiolabeling and/or electrophoretic studies are unobtainable. The cord blood lymphocytes of the male sibling of a WAS patient were collected and analyzed by SEM. The mean score of the cord blood lymphocytes was 2.65 ± 0.14. Further, the neonate's grade 4 and 3 lymphocytes were qualitatively abnormal, with blunted microvilli (Fig 3). The surface morphology of cord blood lymphocytes from two normal neonates could not be distinguished from that of normal peripheral blood lymphocytes (not shown). The patient's cord blood platelet count was 75,000/μL. During the first week of life, his platelet count decreased to 20,000/μL. The patient died of WAS at 4 months of age.

DISCUSSION

The Wiskott-Aldrich Syndrome is a multi-faceted clinical syndrome in which affected males have thrombocytopenia with platelets of reduced size and function, eczema, and immunodeficiency with decreased T lymphocyte function and inability to make antibodies to carbohydrate antigens. The demonstration of glycoprotein abnormalities in both the platelets and lymphocytes of WAS patients[4,5] suggested a common basis for the platelet and T lymphocyte functional deficiencies. The present observations demonstrate that the peripheral blood lymphocytes of WAS patients, like their platelets, are morphologically abnormal.

In the present study, SEM demonstrated that lymphocytes from WAS patients had a marked deficiency of surface microvilli. In agreement with a previous study, >95% of normal lymphocytes were found to be densely covered with microvillus projections.[6] Only 77.8% of WAS lymphocytes displayed the same density of microvillus projections. A scoring system for evaluating surface morphology was established. The mean score for the lymphocytes from 17 of 18 (neonate excluded, see below) WAS patients was below the range of scores of normal lymphocytes. The one patient whose score overlapped with the low normal range (Fig 2) did not differ in any clinical characteristics (degree of immunodeficiency, eczema, or thrombocytopenia) from the other WAS patients.

The immunologic deficiency in WAS is progressive, and the problems of recurrent infections and eczema are the most

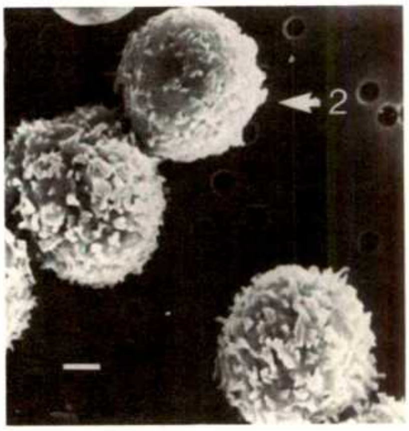

Fig. 3. Cord blood lymphocytes of a WAS patient. The surface microvilli are short and blunt as compared with those of normal lymphocytes (Fig 1A) and differ from the ridgelike projections of thymocytes (Fig 1B). Bar = 1 μm.

variable clinical manifestations and often the last to appear. Of the 18 WAS patients studied (excluding the one neonate), 11 had fully developed eczema and 6 were clinically immunodeficient. Immune abnormalities were detected in most patients, however, by anergy in delayed hypersensitivity skin testing, abnormal circulating T cell phenotypes, poor T cell proliferative responses to specific antigens and mitogens (spontaneous or after immunization), and failure to make antibodies after immunization with specific antigens. In this study, patients were diagnosed as WAS patients based on the dependable criteria of male sex, family history of WAS, and thrombocytopenia with platelets of reduced size. The finding of defective lymphocyte morphology in all these patients suggests that lymphocyte structural alterations represent a previously undescribed and invariable manifestation of the syndrome. This morphological defect is evident early, before clinical immunodeficiency and eczema are manifest, and is independent of splenectomy status and the severity of thrombocytopenia and clinical immunodeficiency. Thus, defective lymphocyte morphology as assessed by SEM represents an additional diagnostic criterion to distinguish WAS in very young or mildly affected patients.

Only 1 to 2 × 10^6 purified lymphocytes are required for SEM. The morphological defect in WAS lymphocytes is independent of the manipulations used to isolate lymphocytes, since SEM analysis of the unfractionated buffy coat layer (at the plasma/red cell interface after gravity sedimentation of whole blood) can detect the presence of morphologically defective WAS lymphocytes (not shown). Therefore, it is possible to evaluate patients from whom only small blood samples are available. SEM was also used to diagnose WAS in one at-risk neonate who could not have been diagnosed on clincial grounds until later. Eventually, it may be possible to apply this diagnostic method to blood samples drawn prenatally.

The relationship between abnormal lymphocyte morphology and immune dysfunction in WAS is not clear. As the simplest interpretation, the lower lymphocyte morphology scores suggest that WAS patients have increased numbers of circulating thymocytes. The surface morphology of WAS lymphocytes, however, with fewer and more blunt surface projections than normal lymphocytes, is qualitatively different from that of thymocytes, which have ridgelike or ruffled surface projections. Furthermore, lymphocyte morphological abnormalities and lower morphology scores were found in all patients whether or not immature T cells could be detected by phenotypic analysis of peripheral blood lymphocytes (not shown). SEM analysis of the lymphocytes of patients with some other primary immunodeficiencies [ataxia telangietasia (2 patients), common variable agammaglobulinemia (1 patient), hyper-IgM syndrome (2 patients), severe combined immune deficiency (2 patients)] has not revealed any morphological lymphoid abnormalities, indicating that altered lymphocyte morphology is not a generalized characteristic of the immunoefficiency state (not shown). Successful bone marrow transplantation corrected the lymphocyte morphological abnormality in three WAS patients studied posttransplant (not shown).

A small percentage of lymphocytes devoid of microvilli are consistently found in normal individuals (ref 6 and Table 1). We hypothesize that the higher percentage of lymphocytes devoid of microvilli in WAS patients results from removal of lymphocyte membrane and represents an accentuation in the patients of the normal mechanisms of lymphocyte turnover. A recent study by Corash and co-workers[7] demonstrated a role for the spleen in an immunologically mediated process that results in the structurally abnormal, small platelets of the WAS. Thus, one interpretation of our findings is that the abnormalities in lymphocyte membrane glycoprotein, gpL115, lead to the splenic removal of membrane fragments and result in the loss of microvillus projections from the lymphocyte surface. The differences in the mean morphology scores of nonsplenectomized and splenectomized WAS patients examined in this study, however, were not statistically significant. We have not yet serially examined lymphocytes of WAS patients after splenectomy, when even a transient improvement in the morphology score would indicate a role for splenic removal of microvilli.

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REFERENCES

1. Wiskott A: Familiarer, angeborener Morbus Welhofii? Monatsschr Kinderheilkd 68:212, 1937

2. Aldrich RA, Steinberg AG, Campbell DC: Pedigree demonstrating a sex-linked recessive condition characterized by draining ears, eczematoid dermatitis and bloody diarrhea. Pediatrics 13:133, 1954

3. Cooper MD, Chase HP, Lowman JT, Krivit W, Good RH: Wiskott-Aldrich syndrome: An immunologic deficiency disease involving the afferent limb of immunity. Am J Med 44:499, 1968

4. Parkman R, Remold-O'Donnell E, Kenney DM, Perrine S, Rosen FS: Surface protein abnormalities in the lymphocytes and platelets from patients with the Wiskott-Aldrich Syndrome. Lancet 2:1387, 1981

5. Remold-O'Donnell E, Kenney DM, Parkman R, Cairns L, Savage B, Rosen FS: Characterization of a human lymphocyte surface sialoglycoprotein that is defective in Wiskott-Aldrich Syndrome. J Exp Med 159:1705, 1984

6. Newell DG, Roath S, Smith JL: The scanning electron microscopy of normal human peripheral blood lymphocytes. Br J Haematol 32:309, 1976

7. Corash L, Shafer B, Blaese RM: Platelet associated immunoglobulin, platelet size and the effect of splenectomy in the Wiskott-Aldrich syndrome. Blood 65:1439, 1985

T Lymphocyte–Derived Differentiation-Inducing Factor Inhibits Proliferation of Leukemic and Normal Hemopoietic Cells

By Urban Gullberg, Eva Nilsson, Mangalasseril G. Sarngadharan, and Inge Olsson

A differentiation-inducing factor (DIF) for the promyelocytic HL-60 cell line is constitutively produced by the malignant T lymphocyte line HUT-102. DIF was highly purified from HUT-102–conditioned media by means of diethylaminoethanol (DEAE)-chromatography, gel chromatography, and high-resolution, ion-exchange chromatography on a MonoQ column and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition to inducing differentiation of wild-type HL-60 cells, resulting in secondary inhibition of growth, DIF, at a tenfold lower concentration, inhibited the growth of some clones of the monoblastic U-937 cell line as well as that of subclones of HL-60. The latter effect was most likely a primary growth inhibition and not secondary to differentiation; 50% inhibition of clonogenic growth in agar was seen at ~1.0 pmol/L of DIF. In addition, the clonogenic growth of fresh leukemia cells from 10 of 12 patients with acute myeloid leukemia (AML) was inhibited with 50% inhibition at ~10 pmol/L of DIF. The growth of normal granulocyte-macrophage colonies was inhibited at a similar concentration, whereas early erythroid colonies were much more resistant. DIF and interferon-γ (γ-IFN) were shown to be separate molecules inasmuch as a neutralizing antibody for γ-IFN did not abolish the DIF effect. The differentiation effect on wild-type HL-60 and the proliferation inhibitory effect on leukemic and normal myeloid cells cochromatographed through all purification steps suggest that both activities are exhibited by identical polypeptides. DIF may have a role in regulating normal hemopoiesis. The growth inhibitory effect of DIF and the ability to induce differentiation of some leukemia cells may suggest a clinical utility in the treatment of leukemia.

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ACUTE MYELOID leukemia (AML) is characterized by an accumulation of immature cells, which may preserve normally transient phenotypes. The block in cell differentiation is not necessarily irreversible, as was shown originally using the murine M-1 myeloid leukemia cell line.[1] Agents that induce differentiation of M-1 cells inhibited leukemia development in mice, indicating that such agents can act in vivo.[2] Furthermore, injection of M-1 cells into embryos can result in the appearance in adult mice of normal granulocytes partially derived from the leukemic clone,[3] indicating that the malignant cells respond to normal differentiation control in the embryo. We[4,5] and others[6,7] demonstrated that mitogen-stimulated human mononuclear blood cells release polypeptides called differentiation-inducing factors, DIFs,[4,5] which can induce the promyelocytic HL-60 line[8] to mature into monocytelike cells. Subsequently, we showed that the T lymphocyte leukemic cell line HUT-102, which releases human T cell leukemia virus (HTLV),[9] displayed constitutive production of DIF.[10] DIF has been partly characterized[10]; it has a mol wt of ~50,000. It acts synergistically with retinoic acid to induce differentiation of HL-60.[10]

Now, we report that DIF, in addition to its differentiation effect on HL-60 cells, has a potent growth inhibitory effect on other myeloid cell lines and on fresh clonogenic cells from patients with AML. Normal granulocyte-macrophage clonogenic precursors (CFU-GM) and erythroid progenitors (BFU-E) are also susceptible. Thus, the ability of the same agent both to inhibit self-renewal and to induce the process of differentiation of some leukemic cells is emphasized.

MATERIALS AND METHODS

Chemicals

12-*O*-Tetradecanoyl phorbol 13-acetate (TPA) and Nitroblue tetrazolium (NBT), were from Sigma Chemical Co (St Louis). Acrylamide/*bis*-acrylamide (29:1) was from Bio-Rad (Richmond, CA). The Bolton-Hunter reagent, [^{125}I]-4, hydroxyphenylpropionic acid was from New England Nuclear, Dreieich, FRG. Diethylaminoethanol (DEAE)-Sephacel, Sephadex G-75, MonoQ columns, and Superose columns were from Pharmacia Fine Chemicals (Uppsala, Sweden). Luxol fast blue was from T.T. Gurr, High Wycombe, Bucks, England.

Cell Lines

The HL-60,[8] U-937,[11] K-562,[12] and KG1[13] cell lines were maintained in suspension culture in RPMI 1640 medium containing 10% fetal bovine serum (FBS). The same medium was used for agar cultures. Exponentially growing cells were used for assays. Subclones of cell lines were obtained by picking individual colonies from agar cultures and expanding the cells by incubation in suspension culture.

Assay for DIF

The differentiation effect was assayed as previously described.[10] In brief, cells were exposed to DIF in suspension culture for 4 days, and the number of unspecific esterase-positive and NBT-positive cells were determined. One unit of DIF is defined as the amount necessary to increase by 10% the number of NBT-positive cells. The effect of DIF on growth of cell lines was determined both in suspension and in agar culture. In the latter case, 2,000 cells were seeded in 0.3% agar, and colonies were counted after 7 days.

Assay for CFU-GM

Human marrow cells (10^5) (density <1.077 g/mL) obtained by centrifugation in Isopaque-Ficoll were cultured in 1 mL of 0.3% agar on top of 1 mL of 0.5% agar in McCoy's medium with 15% FBS in

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35-mm tissue culture dishes. As a source of colony-stimulating activity (CSA), human placenta conditioned medium (HPCM) was used[14] after partial purification by chromatography on Sephadex G-75. For colony morphology studies, agar gels were fixed in 5% glutaraldehyde and dried; they were then stained with 0.1% Luxol fast blue and counterstained with Harris hematoxylin.

Assay for BFU-E

Mononuclear blood cells of four healthy subjects were isolated from heparinized blood by centrifugation on Ficoll-Hypaque and cultured at 2×10^5/mL in 0.8% methyl cellulose in Iscove's modification of Dulbecco's medium, supplemented with 30% FBS, 20 mmol/L of glutamine, 50 μmol/L of β-mercaptoethanol, 1.7% bovine serum albumin (BSA), 1.6 μmol/L of $FeCl_3$ and 4.5 μmol/L of transferrin. Ten percent conditioned medium from the Mo cell line was used as CSA together with 1 U/mL of human urinary erythropoietin, kindly provided by Dr David Golde of the Department of Oncology-Hematology, University of California at Los Angeles, and Dr Miloslav Beran, M.D. Anderson Hospital, Houston, respectively. The BFU-E were counted after 20 days. Control incubations without DIF contained 41 ± 25(SD) BFU-E per dish.

Interferon-γ (γ-IFN) and Anti–γ-IFN

Recombinant γ-IFN (Ernst-Boehringer Institut für Arzneimittelforschung) contained $\sim 2 \times 10^7$ IFN U/mg of protein. A murine IgG monoclonal antibody that neutralized the antiviral and anticellular effects of γ-IFN was obtained from the same source. Neutralizing anti–γ-IFN antibody was incubated with DIF overnight before testing.

Production of Conditioned Medium

Roller bottle cultures of HUT-102, clone B2, grown in RPMI, 10% FBS, were harvested at 96 hours postseeding. Supernatant was clarified by centrifugation at 1,600 rpm for 20 minutes and run through a continuous flow Model K ultracentrifuge (Electro-Nucleonics, Rockville, MD) at 35,000 rpm at a rate of 10 to 11 L/h on a sucrose step gradient (20% to 60% in 10 mmol/L of Tris, pH 7.2, 0.1 mol/L of NaCl, 1 mmol/L of EDTA). The virus-free effluent (LyCM) was collected and stored at 4 °C.

Isolation of DIF

DIF was isolated from 40 L of conditioned medium, which was concentrated by ultrafiltration using a Pellicon casette (Millipore, Bedford, MA) with a filter of a cut-off point at a mol wt of 10,000. Dialysis was carried out against 10 mmol/L of Tris-HCl buffer, pH 8.0, containing 0.1 mmol/L of phenylmethylsulfonylfluoride (PMSF), 0.01% polyethylene glycol (PEG) 6,000, and 1 mmol/L of dithiothreitol (buffer A).

Step 1: DEAE-Sephacel chromatography. The concentrated and dialyzed conditioned medium was applied in three separate runs on a 360-mL column equilibrated with buffer A. Elution was performed with an 800-mL NaCl/buffer A gradient, ranging from 0 to 0.3 mol/L of NaCl. The DIF eluted at 0.05 to 0.10 mol/L of NaCl was pooled, dialyzed against buffer A, and rechromatographed once on DEAE-Sephacel.

Step 2: Gel chromatography. DIF-containing material from step 1 was subjected to gel chromatography (four separate runs) on a Sephadex G-75 superfine column (5 × 100 cm) eluted with 0.15 mol/L of NaCl, buffer A. DIF-containing fractions were pooled and concentrated by ultrafiltration and rechromatographed once.

Step 3: High-resolution, ion-exchange chromatography. DIF from step 2 was subjected to chromatography on a MonoQ column (1 × 10 cm) equilibrated with buffer A. Elution was performed with a 60-mL NaCl/buffer A gradient, ranging from 0 to 0.2 mol/L of NaCl using the Fast-Pressure Liquid Chromatography (FPLC) system (Pharmacia). DIF eluted at 0.05 to 0.10 mol/L of NaCl was rechromatographed, and only the DIF eluted at <0.08 mol/L of NaCl was recovered to increase the specific activity.

Step 4: SDS-PAGE. SDS-PAGE was performed as described[15] using 1.5-mm slab gels of 5% to 20% acrylamide. DIF-containing fractions from step 3 were concentrated in a dialysis bag placed inside a heap of solid PEG-20,000. Electrophoresis sample buffer[15] was added, and the mixture containing 50 mmol/L of Tris HCl buffer, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 50 mmol/L of PMSF, and 20% glycerol, was incubated at 60°C for 20 minutes. After electrophoresis, part of the gel was stained to localize mol wt standards: albumin (mol wt 67,000), ovalbumin (mol wt 43,000), Carbanhydrase (mol wt 30,000), soya bean trypsin inhibitor (mol wt 20,000), and α-lactalbumin (mol wt 14,400). The unstained gel matrix was sliced into 0.25-cm strips. The slices were extracted overnight with 1 mL 5% FBS in 0.15 mol/L of NaCl, dialyzed against 0.15 mol/L of NaCl, buffer A, and assayed for biological activity. Some DIF-containing material of step 3 was also iodinated using the Bolton Hunter reagent according to the description of the manufacturer. After electrophoresis, gels were dried on filter paper and exposed to x-ray film (Kodak X-Omat) for 12 hours.

Other Chromatographic Methods

A Superose 12 column was used for gel filtration equilibrated with 0.15 mol/L of NaCl, buffer A. Protein was measured using the Bio-Rad protein assay or estimated from the absorbance of 280 nm; an absorbance of 1.0 was considered to correspond to 1 mg/mL of DIF.

RESULTS

Purification of DIF From HUT-102 Conditioned Medium

The purification steps are modifications of procedures used in a previous report on the biochemical characterization of DIF.[10] Especially high-resolution ion-exchange chromatography of the present work represented a powerful improvement. Table 1 summarizes data on the yield and specific activities from each step of purification. DIF-containing fractions obtained by MonoQ chromatography (step 3) showed a 37,000-fold purification with an overall

Table 1. Purification of DIF From 40-L HUT-102 Conditioned Medium

Fraction	Protein	Total Activity ($U \times 10^3$)	Specific Activity (U/mg)	Purification (-Fold)	Yield (%)
HUT-102 medium	195 g	80	0.41	1.0	100
DEAE-Sephacel	6.25 g	64.4	10.3	25	81
Sephadex G-75	0.44 g	48	109.1	266	60
MonoQ I	64 mg	40.8	637.5	1,554	51
MonoQ II*	1.6 mg	24.4	15,250	37,195	31
SDS-PAGE†	—	3.8	—	—	5

DIF, differentiation-inducing factor; HUT-102, malignant T lymphocyte cell line; DEAE, diethylaminoethanol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

*MonoQ II refers to rechromatography on MonoQ followed by collection of DIF eluted at <0.08 mol/L of NaCl to increase the specific activity.

†The protein content of fractions eluted from the gel was too low to be determined.

yield of 31% and were used in experiments described below unless otherwise stated. Concentrations of DIF are given as total protein added of this fraction. From experiments with radioiodination using the Bolton-Hunter reagent followed by SDS-PAGE (Fig 1) and elution of DIF from the gel ~5% of the radioiodinated protein was calculated to be associated with DIF activity. Estimations of molar concentrations of DIF which follow are based on these data and a mol wt of 50,000.

Proliferation Inhibitory Effects on Myeloid Leukemia Cell Lines

Induction of ~50% of wild-type HL-60 into NBT-positive, α-naphthyl butyrate esterase-positive monocytelike cells was achieved with ~50 pmol/L of DIF. This represents the characteristic differentiation-inducing effect of DIF reported earlier.[10] The results reported here demonstrate that at a tenfold lower concentration, DIF inhibited the growth of some subclones of both U-937 and HL-60 without the production of mature NBT-positive cells. Figure 2 shows a dose-dependent inhibitory effect of DIF on proliferation of a subclone of U-937[16] in suspension culture (top panel). The antiproliferative effect was partly reversible; when the cells were exposed to DIF for 3 days, washed, and resuspended in fresh medium, a slow recovery of growth occurred, indicating that at least a fraction of the cells had survived (Fig 2, middle panel). The growth inhibitory effect (like the differentiation effect)[10] was rather heat stable but was destroyed by boiling (Fig 2, lower panel). The growth of a subclone of HL-60 (HL-60-10) was inhibited at similar concentrations (data not shown). In contrast to wild-type HL-60, the subclone was α-naphthyl butyrate esterase-positive and thus similar to the monoblastlike U-937.

The growth inhibitory effect of DIF was more pronounced using a clonogenic assay instead of suspension culture. The growth inhibitory effect of DIF on the clonogenic growth in agar of various myeloid leukemic cell lines was compared (Table 2). HL-60-10, U-937, and KG-1 showed a similar susceptibility to DIF, whereas the K-562 cell line showed a tenfold lower susceptibility.

The addition of 4,000 neutralizing units of anti-γ-IFN antibody did not neutralize the proliferation inhibitory effect

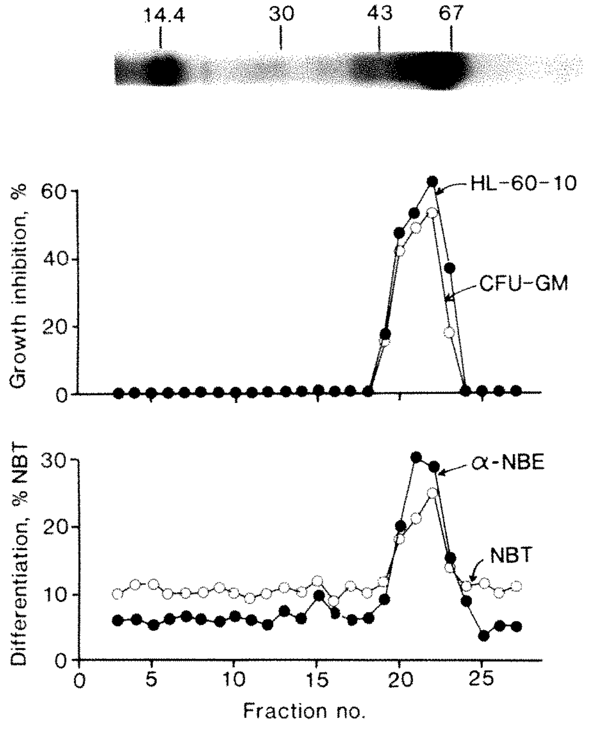

Fig 1. Copurification of differentiation and antiproliferative effects of DIF on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Three hundred units of DIF obtained in step 3 (MonoQ chromatography) were subjected to SDS-PAGE. Of eluted fractions, 100 μL were used for assay of differentiation induction of wild-type HL-60, 50 μL were used for assay of inhibition of clonogenic growth of CFU-GM, and 20 μL were used for assay of inhibition of clonogenic growth of a subclone of HL-60 (HL-60-10). ^{125}I-Labeled MonoQ DIF was electrophoresed simultaneously, and this part of the gel was dried and autoradiographed; it is shown (top) with mol-wt markers. The position of various activities eluted from the gel are given. In addition, some ^{125}I-labeled DIF was added to the nonlabeled DIF before electrophoresis; by measuring the radioactivity of the fractions eluted after electrophoresis, it was possible to make an estimate of how much of the radioiodinated protein corresponded to DIF.

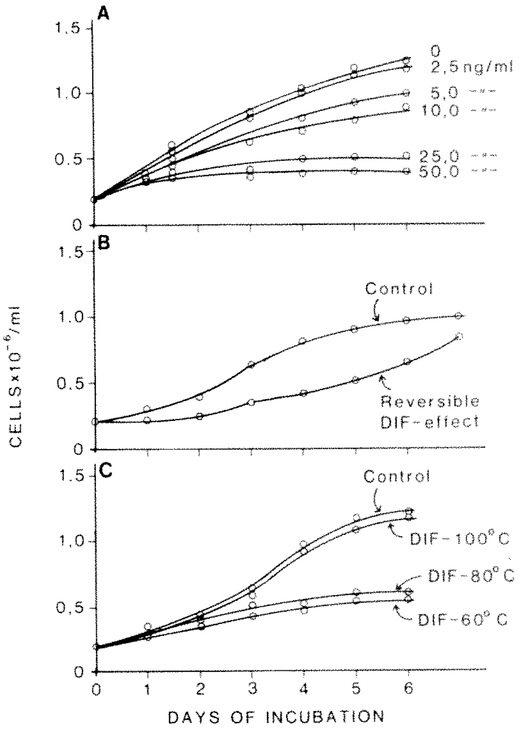

Fig 2. Effect of DIF on the proliferation of U-937 in suspension culture. DIF from step 3 (MonoQ chromatography) was added to the cultures, and cell counts were determined during 6 days. Top panel: growth inhibitory effects of various concentrations of DIF. Middle panel: partial reversibility of the antiproliferative DIF effect; cells were incubated without (control) or with 50 ng DIF/mL for 3 days and were washed and resuspended in fresh medium, and the proliferation was monitored. Bottom panel: heat sensitivity of DIF using 10 ng/mL of DIF heat-treated for 20 minutes.

Table 2. The Growth Inhibitory Effect of DIF on Clonogenic Growth in Agar of Myeloid Leukemic Cell Lines

Cell Line	50% Growth Inhibition (ng/mL DIF)*
HL-60 (wild type)	>100
HL-60-10	1
HL-937	1
KG-1	2
K-562	11

Differentiation-inducing factor (DIF) from step 3 (MonoQ chromatography) was used for these assays.

of DIF (Fig 3). Identical results were also obtained when immunoprecipitates, which might have formed with the antibody, were removed by adsorption to protein A-Sepharose prior to testing. Furthermore, γ-IFN 100 to 4,000 U/mL, unlike DIF, did not induce any maturation of wild-type HL-60 as judged by lack of induction of NBT-positive cells (data not shown). Treatment at pH 2 only partially reduced the DIF activity (Fig 3). All these results indicate that DIF and γ-IFN are nonidentical.

Proliferation Inhibitory Effects on Normal Hemopoietic Clonogenic Cells and on Fresh AML Cells

Figure 4 shows that normal CFU-GM and BFU-E as well as clonogenic fresh AML-cells from 10 of 12 patients responded with growth inhibition to the addition of DIF. CFU-GM counted on day 14 were more susceptible than were CFU-GM counted on day 10, whereas cluster-forming cells counted on day 7 were even less susceptible. Only a 20% inhibition of growth of BFU-E was documented over a wide range of DIF concentrations. Actually, a significant stimulation of the growth of BFU-E was demonstrated at very low concentrations. Effects on CFU-GMs at such low concentrations of DIF were not investigated. A 50% growth inhibition of both CFU-GM (day 10) and AML cells was seen at ~10 pmol/L of DIF (Fig 4). Treatment with DIF did not change the commitment of CFU-GM inasmuch as the distribution of macrophages and granulocytes in residual colonies did not differ from that of control cultures (data not shown). Like the antiproliferative effect of DIF on leukemic cell lines, the effect on normal CFU-GM was rather heat resistant, resistant to a neutralizing antibody against γ-IFN, and only partially susceptible to low pH (Fig 5).

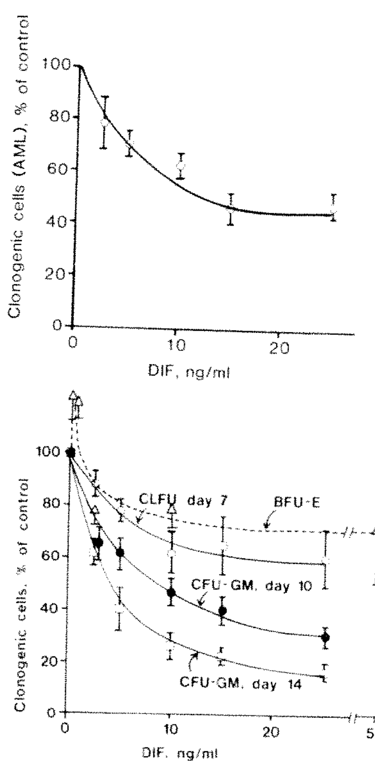

Fig 4. Effects of DIF on clonogenic growth of bone marrow cells from ten patients with acute myeloid leukemia (AML), normal CFU-GM of marrow cells, and normal BFU-E of mononuclear blood cells. DIF from step 3 (MonoQ chromatography) was used. The plating efficiency of marrow cells from the AML patients varied between 0.3% and 2.0%. Cells from two patients with AML were resistent to DIF, and results for these were excluded. Growth of clonogenic AML cells was read at day 7. Cluster-forming units (CLFU) were read at day 7. CFU-GM were read at days 10 and 14, and BFU-E were read at day 20. Bars, SEM.

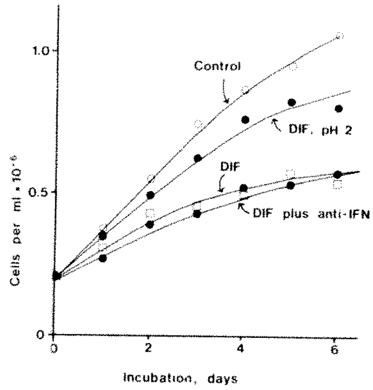

Fig 3. Effect of low pH and a neutralizing anti-γ-IFN antibody on the proliferation inhibitory effect of DIF on U-937. DIF was treated at pH 2 or with 4,000 neutralizing units of antibody overnight prior to testing. Control shows growth of cells without added DIF.

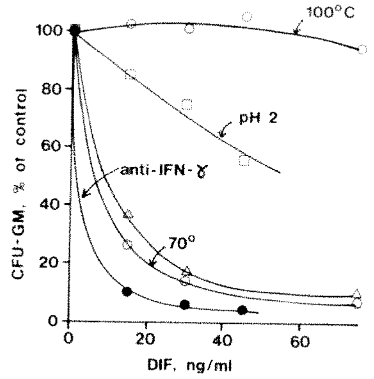

Fig 5. Effect of heat treatment, low pH, and a neutralizing anti-γ-IFN antibody on the proliferation inhibitory effect of DIF on normal CFU-GM. Control incubation with DIF (Δ). DIF was treated at pH 2 (□) or with 4,000 neutralizing units of the antibody (●) overnight prior to testing. Heat treatment was for 20 minutes at 70 °C and 100 °C (○).

Copurification of Differentiation-Inducing Effect and Proliferation Inhibitory Effect of DIF

Both activities cochromatographed exactly on DEAE chromatography (step 1). When material from step 3 was subjected to rechromatography on a MonoQ column or on a Superose column, the activities copurified (Fig 6). Some molecular heterogeneity is indicated by the elution pattern from the MonoQ column. This is consistent with results from chromatofocusing demonstrating that the isoelectric point varied between pH 5.4 and 5.9 (data not shown). In addition, when the material from step 3 was subjected to SDS-PAGE followed by elution of gel slices, both activities were found in the same fractions (Fig 1) and a mol wt of ~50,000 was calculated. By addition of some ^{125}I-labeled DIF prior to electrophoresis, it was estimated that 5% of the protein content of the MonoQ fraction may consist of DIF.

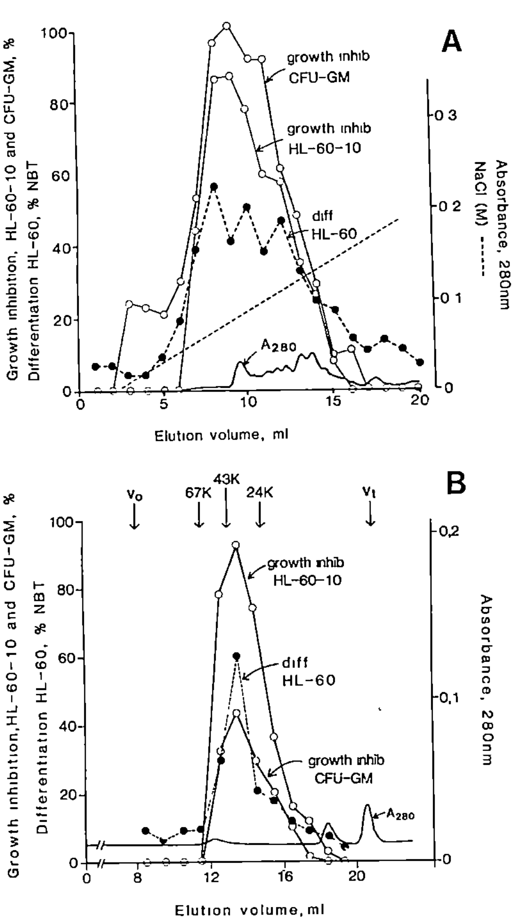

Fig 6. Copurification of differentiation and antiproliferative effects of DIF on a MonoQ column and a Superose column. Six hundred units of DIF obtained in step 3 were rechromatographed on a 1-mL MonoQ column (A) eluted with 1-mL fractions using a gradient of NaCl-buffer A. Forty microliters were used for assay of differentiation induction of wild-type HL-60, 10 µL were used for assay of inhibition of clonogenic growth of CFU-GM, and 0.5 µL were used for assay of inhibition of clonogenic growth of a subclone of HL-60. Three hundred units of DIF obtained in step 3 were chromatographed on a Superose 12 column (B) eluted with 1-mL fractions using 0.15 mol/L of NaCl-buffer A. Eighty microliters were used for assay of differentiation induction of wild-type HL-60, 10 µL were used for assay of inhibition of clonogenic growth of CFU-GM, and 10 µL were used for assay of inhibition of clonogenic growth of a subclone of HL-60 (HL-60-10).

DISCUSSION

DIF induces the differentiation of wild-type HL-60 along the monocyte-macrophage pathway,[5,10] resulting in secondary inhibiton of growth. In addition, results from this report show that, at a tenfold lower concentration, DIF inhibits growth of subclones of both HL-60 and U-937. The latter effect was expressed without visible differentiation, as judged by the lack of production of NBT-positive cells characteristic of the mature differentiation product.[4-7,10] Even if phenotypic modulation may have occurred, it is unlikely that the rapid antiproliferative effect observed was secondary to induction of differentiation in this case. Thus, DIF appears to display different actions on clones from the same cell line. Target cells that are resistant to a growth inhibitory effect at a low concentration may respond with differentiation at a higher concentration of DIF as wild-type HL-60 cells do. In both cases, the final result will be inhibition of the self-renewal capacity of the leukemic cells.

In a cell line, a large fraction of cells has a capacity for clonal self-renewal. Among fresh leukemia cells from patients with AML only a minority of cells are clonogenic, however. Therapeutic efforts must be directed toward the clonogenic leukemia cells becasue nonclonogenic cells will disappear anyway. The growth inhibition by DIF of marrow cells from patients with AML using a clonal assay may suggest a utility of DIF in the treatment of leukemia. The effect on AML cells may be a direct inhibition of proliferation of leukemic clonogenic cells or may be secondary to induced differentiation. The plating efficiency in agar of marrow cells from patients with AML varied considerably,[17] and it was a parameter of prognosis inasmuch as a high plating efficiency indicated a less favorable prognosis.[18] For natural reasons, the antiproliferative effect of DIF could only be significantly evaluated on AML cells with a relatively high plating efficiency. Therefore, the patient material investigated became selected to include patients with a less favorable prognosis preferentially. On the other hand, in such cases with high plating efficiency one can be confident that the majority of clonogenic cells really represents leukemic and not normal cells.

The growth inhibitory effect on normal CFU-GM suggests that DIF may be involved in a physiological regulation of hemopoiesis. It is important to determine if DIF also has effects on multipotent (mixed) progenitors (CFU-GEMM), especially if DIF is considered as a treatment in leukemia. Assaying the effects of DIF on multipotent progenitors was difficult, however, because daughter cells of such cells are CFU-GM, which are susceptible to the growth inhibitory effects of DIF

As far as we can judge, DIF is not identical with previously described lymphokines. Interferons affect many cell functions in addition to their inhibitory action on virus replication. Among these effects are proliferation inhibitory effects on hemopoietic cells in vitro[19] and differentiation-inducing effects on leukemic cells.[20,21] An identity between DIF and γ-IFN was ruled out, however, because a neutralizing antibody to γ-IFN did not interfere with the proliferation inhibitory effect of DIF. Furthermore, in contrast to DIF, recombinant γ-IFN did not induce differentiation of wild-type HL-60 judged by lack of production of NBT-positive cells Whether DIF is a unique differentiation and cytotoxic protein produced by lymphocytes or is related to the family of polypeptides called lymphotoxins remains to be investigated.[22]

That both the differentiation and the antiproliferative effects are exhibited by the same polypeptide(s) is strongly suggested since both activities cochromatographed during all purification steps. Furthermore, the yield of both activities from various purification procedures was similar (data not shown). A purification to homogeneity has not been achieved, however, and it has not been shown that completely purified DIF displays both activities.

Constitutive production of DIF by the HUT-102 line is a valuable source for large-scale production and for isolation and cloning of the gene that codes for DIF. An agent like DIF that can both inhibit self-renewal and induce differentiation of some leukemic cells is of potential utility in the treatment of leukemia.

REFERENCES

1 Sachs L: Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. Nature 274:535, 1978

2. Lotem J, Sachs L: In vivo inhibition of the development of myeloid leukemia by injection of macrophage-granulocyte-inducing protein. Int J Cancer 28:375, 1981

3. Gootwine E, Webb C, Sachs L: Participation of myeloid leukaemic cells injected into embryos in haemopoietic differentiation in adult mice. Nature 299·63, 1982

4 Olsson I, Olofsson T. Induction of differentiation in a human promyelocytic leukemic cell line (HL-60). Production of granule proteins. Exp Cell Res 131.225, 1981

5 Olsson I, Olofsson T, Mauritzon N Characterization of mononuclear blood cell derived differentiation inducing factors (DIFs) for the human promyelocytic leukemic cell line HL-60 J Natl Cancer Inst 67:1225, 1981

6. Chiao JW, Freitag WF, Steinmetz JC, Andreff M: Changes of cellular markers during differentiation of HL-60 promyelocytes to macrophages as induced by T lymphocyte conditioned medium. Leuk Res 5:477, 1981

7. Leung K, Chiao JW: Human leukemia cell maturation induced by T-cell lymphokine isolated from medium conditioned by normal lymphocytes Proc Natl Acad Sci USA 82.1209, 1985

8. Collins SJ, Gallo RC, Gallagher RE. Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature (Lond) 270·347, 1977

9. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Isolation of type-C retrovirus particles from cultured and fresh lymphocytes of a patient with cutaneous T-cell lymphoma Proc Natl Acad Sci USA 77.7415, 1980

10. Olsson I, Sarngadharan MC, Breitman TR, Gallo RC Isolation and characterization of a T-lymphocyte derived differentiation inducing factor (DIF) for myeloid leukemic cells. Blood 63:510, 1984

11. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell-line (U-937) Int J Cancer 7.565, 1976

12. Lozzio CB, Lozzio BB: Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45:321, 1975

13 Koeffler CB, Golde DW: Acute myelogenous leukemia A human cell line responsive to colony-stimulating activity. Science 200.1153, 1978

14 Burgess AW, Wilson EMA, Metcalf D: Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood 49 573, 1977

15. Bonner WM, West MHP, Stedman JD. Two-dimension gel analysis of histones in acid extracts of nuclei, cells, and tissues. Eur J Biochem 109.17, 1980

16. Olsson I, Gullberg U, Ivhed I, Nilsson K. Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1,25-dihydroxycholecalciferol. Cancer Res 53:5862, 1983

17 Moore MAS, Spitzer G, Williams N, Metcalf D, Buckley J: Agar culture studies in 127 cases of untreated acute leukemia: The prognostic value of reclassification of leukemia according to in vitro growth characteristics. Blood 44.1, 1974

18 Gustavsson A, Mitelman F, Olofsson T, Olsson I. The relationship between growth in agar, karyotype and prognosis in acute non-lymphocytic leukemia Scand J Haematol 32:351, 1984

19 Broxmeyer H, Lu L, Platzer E, Feit C, Juliano L, Rubin B. Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells J Immunol 131:1300, 1983

20. Tomida M, Yamamoto Y, Hozumi M. Stimulation of induction of differentiation of human promyelocytic leukemia cells Biochem Biophys Res Commun 104:30, 1982

21 Ralph P, Harris PE, Punjabi CJ, Welte K, Litcofsky PB, Ho M-K, Rubin BY, Moore MAS, Springer IA: Lymphokine inducing "terminal" differentiation of the human monoblast leukemia line U-937: a role for γ-interferon. Blood 62:1169, 1983

22. Aggarwal BB, Henzel WJ, Moffat B, Kohr WJ, Harkins RN: Primary structure of human lymphotoxin derived from 1788 lymphoblastoid cell line J Biol Chem 260:2334, 1985

Human Gamma Interferon Enhances Release From Phytohemagglutinin-Stimulated T4$^+$ Lymphocytes of Activities That Stimulate Colony Formation by Granulocyte-Macrophage, Erythroid, and Multipotential Progenitor Cells

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Human gamma interferon (HuIFNγ) was evaluated for its effects on the release from human peripheral blood T lymphocytes (>98% pure) stimulated by phytohemagglutinin (PHA) of activities that can stimulate granolocyte-macrophage (CFU-GM) colonies and clusters, erythroid (BFU-E) bursts, and mixed (CFU-GEMM) colonies. T lymphocytes did not release these activities in the absence of PHA with or without HuIFNγ. In the presence of PHA, pure natural HuIFNγ at concentrations of 0.1 to 100 U/mL significantly enhanced release of these colony-stimulating activities. Although enhanced release of granulocyte-macrophage colony-stimulating activities were noted when T lymphocytes were added to the conditioning medium in the presence of 0.1%, 0.5%, and 1% PHA, enhanced release of burst-promoting and mixed colony activities was seen only in the presence of 0.1% and 0.5% PHA. The enhanced release of colony-stimulating activities was not due to HuIFNγ-suppression of the release from PHA-stimulated T lymphocytes of suppressor molecules. The enhancing effects of natural HuIFNγ were neutralized with a monoclonal anti-natural HuIFNγ, and recombinant HuIFNγ mimicked the enhancing effects of the natural HuIFNγ. This enhancing effect was noted only when HuIFNγ was added with the T lymphocytes and PHA during the first 24 hours of incubation. T lymphocytes were separated into T4$^+$, T8$^-$, T8$^+$, and T4$^-$ subsets (>98% pure for the appropriate phenotypes) after incubation with OKT4$^-$ and OKT8$^-$ monoclonal antibodies and sorting on a fluorescence-activated cell sorter (FACS). All types of colony-stimulating activities were released from each population after stimulation with PHA, but enhanced release of these activities in the presence of HuIFNγ was only detected with the T4$^+$ or T8$^-$ subsets of lymphocytes. It cannot be concluded from these studies whether HuIFNγ is enhancing the release of one or several types of colony-stimulating activities, but these studies suggest a role for HuIFNγ and T4$^+$ lymphocyte subsets in the regulation in vitro of the release of colony-stimulating activities.

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HEMATOPOIETIC progenitor cells require the presence of specific growth factors for their proliferation and differentiation in vitro.[1-4] Granulocyte-macrophage progenitor cells (CFU-GM) require the action of granulocyte macrophage (GM) colony-stimulating factor (CSF) for their proliferation and differentiation; primitive erythroid progenitors, the burst-forming units (BFU-E) are dependent on burst-promoting activity (BPA) for early growth in erythropoietin-supplemented cultures; and multipotential progenitors (CFU-GEMM) require multi-CSF or interleukin-3 (IL-3) in addition to erythropoietin.

GM-CSF, BPA, and multi-CSF (IL-3) are produced by various cell types and by interactions between these cells. Cell types implicated in the production/release of these factors include monocytes, macrophages, T lymphocytes, endothelial cells, fibroblasts, and other cells.[5-25] Purified populations of T lymphocytes are capable of releasing these forms of CSF when cells are activated by mitogens or antigens,[26-32] and both T4$^+$ and T8$^+$ lymphocyte subsets produce GM-CSF and BPA.[33-35]

Interferons are a family of inducible secretory proteins usually produced by cells in response to viruses and other stimuli.[36,37] The interferons have been demonstrated to have multiple functions, including the capacity to suppress colony formation by hematopoietic progenitor cells,[38-40] to induce protein synthesis,[41] and the expression of major histocompatibility (MHC) class II antigens on cells,[42-47] to make cells responsive to the suppressive effects of other molecules,[46,47] and to enhance the release of GM-CSF from monocytes.[10,48,49]

In this study, we investigated the effect of HuIFNγ on the capability of highly purified T lymphocytes and their subsets to release GM-CSF, BPA, and multi-CSF type activities when stimulated with phytohemagglutinin (PHA).

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MATERIALS AND METHODS

Specimen collection. Peripheral blood and bone marrow cells were collected in preservative-free heparin from healthy volunteers after they gave their informed consent.

Cell separation procedure. Intact mononuclear blood cell suspensions were separated by density-gradient centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Monocytes were removed by adherence to plastic dishes. Nonadherent cells were collected by gently swirling the dishes and slowly pipetting off the medium. To enrich for T lymphocytes, nonadherent mononuclear cells were rosetted with neuraminidase-treated sheep red blood cells (SRBC).[10] Rosettes were separated by centrifugation on Ficoll Hypaque, and T cells were recovered by red cell lysis with ammonium chloride, pH 7.2. This fraction contained <1% monocytes (as detected by nonspecific esterase staining) and >98% T cells, as determined by fluorescence-activated cell sorter (FACS) analysis using the OKT11a (anti-sheep erythrocyte) pan-T cell antibody or the OKT3 pan-T cell antibody (Ortho Diagnostic, Raritan, NJ) T cell subsets were purified from low-density nonadherent E rosetting cells (E$^+$ cells) under sterile conditions by a FACS IV or FACS 440 (Becton Dickinson, Sunnyvale CA), as described previously for other antibodies,[10] using the murine monoclonal antibodies OKT4 and OKT8 (Ortho Diagnostics). Cells from the T cell-enriched suspensions were incubated in 0.5 mL of RPMI culture medium (GIBCO, Grand Island, NY) + 15% fetal bovine serum (FBS) (Hyclone, Logan, UT) containing OKT4 and OKT8 at final dilutions of 1:100 for 40 minutes at 4°C. The cells were washed twice with ice-cold medium and then incubated with fluorescein-labeled rabbit antimurine IgG F(ab')$_2$ fragments (Cappel Laboratories, Westchester, PA) for 30 minutes on ice. After three washes, the cells were suspended in phosphate-buffered saline (PBS) + 1% FBS at concentrations of $\sim 3 \times 10^6$/mL. They were then sorted with a FACS under sterile conditions into two lymphocyte subpopulations. those binding the monoclonal antibody (T4$^+$ or T8$^+$) and those failing to do so (T4$^-$ and T8$^-$) At the end of the separation run, a sample of each isolated subpopulation of cells was analyzed on the FACS to determine the percentage of cells falling within the original respective sorting gates The T4$^+$ fraction contained >98% positive cells; the T4$^-$ fraction contained <0.5% positive cells. The T8$^+$ and T8$^-$ fraction contained >98% and <1% positive cells, respectively Viability (assessed by Trypan Blue dye exclusion) was always >95% following sorting

Preparation of conditioned medium (CM) to be assessed for release of GM-CSF and BPA Intact T lymphocyte preparations, isolated as described above, were suspended in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS at a concentration of 1×10^6/mL with 0.1% to 1.0% PHA (PHA H15 Wellcome, Beckenham, England) with and without indomethacin 10^{-6}mol/L (Sigma Chemical Co, St Louis) and in the absence or presence of varying concentrations of natural (2×10^7 NIH reference units per milligram) or recombinant ($\sim 5 \times 10^6$ U/mg) HuIFNγ The natural HuIFNγ was produced by stimulation of human peripheral blood leukocytes with *Staphylococcus* enterotoxin A, and recombinant HuIFNγ was a generous gift from Dr H Michael Shepard, Genentech, San Francisco. Unless otherwise noted, the natural HuIFNγ was used. The suspensions were cultured at 37 °C in a humidified atmosphere with 5% CO$_2$ in air for 5 days, this period having been previously determined to be optimal for release of colony-stimulating activities. The cell-free supernatant media were then harvested and kept frozen until assayed for GM-CSF, BPA, and multi-CSF type activities. Sorted T4$^+$, T4$^-$, T8$^+$, and T8$^-$ cells were subjected to the same procedure Indomethacin was used in some of the experiments to prevent the possible endogenous production of prostaglandins because E-type prostaglandins suppress colony formation by CFU-GM[50] and enhance colony formation by BFU-E.[51] To neutralize the activity of the natural HuIFNγ during the conditioning medium period, or to neutralize the carry-over effects of HuIFNγ into the target assay, the natural HuIFNγ or the CM containing or not containing HuIFNγ were preincubated with monoclonal antibody against natural HuIFNγ[41] for 90 minutes at room temperature prior to assay.

Preparation of bone marrow cells Mononuclear cells were separated on Ficoll-Hypaque gradients, washed three times, and resuspended in IMDM with 10% FBS. In some experiments, T lymphocytes and monocytes were removed from bone marrow preparations by incubation for 90 minutes at 37 °C in plastic dishes and subsequent E rosetting of nonadherent cells. In these preparations, monocytes were <1% and T cells were <0.5% of the cell populations

Colony-stimulating activity assays. GM-CSF was quantified in agar and BPA and multi-CSF were quantitated in methylcellulose cultures as described elsewhere. For assay of CFU-GM, bone marrow cells were plated in triplicate at 1×10^5 low-density cells/mL or 3×10^4 nonadherent low-density T lymphocyte-depleted cells/mL in 35-mm Petri culture dishes containing a 1-mL mixture of IMDM, 10% FBS, 0.3% agar, and 10% (vol/vol) of the cell-free supernatant medium to be tested for GM-CSF, as the sole source of exogenously added GM-CSF. In some cases, T lymphocyte-CM was assessed in the presence of 5637 CM.[52] When bone marrow target cells were devoid of accessory cells potentially producing GM-CSF during the assay, no colony or cluster formation was observed without the addition of lymphocyte CM or 5637 CM When intact mononuclear bone marrow cells were used, only a few small aggregates composed of three to ten cells were observed without exogenously added CSF. Cultures were incubated for 10 days at 37 °C in a humidified atmosphere of 5% CO$_2$ in air or at lowered (5%) O$_2$ tension (Oxyreducer, Reming Bioinstruments, Syracuse, NY). Results are expressed mainly as colonies plus clusters, since this is a more accurate estimation of the actual number of progenitor cells stimulated than is the number of colonies alone[53] but, as described in the text, results for colonies were similar to those noted for colonies plus clusters. For colony morphology, agar cultures were overlaid with 1 mL of 0.5% glutaraldehyde (Sigma) in 0.1 mol/L of phosphate buffer After 6 minutes, glutaraldehyde was removed, and 2 mL of absolute methanol was added to the plates. After the methanol evaporated (12 to 18 hours) the fixed cultures were submerged in distilled water After rehydration, the agar pellicles were floated onto clean glass slides and allowed to dry For identification of granulocytes and monocytes/macrophages, colonies were stained for 45 minutes with α-naphthyl acetate esterase (Sigma Kit No. 90-A1) and counterstained with Mayer's hemotoxylin for 10 minutes. Slides were washed, dried at room temperature, mounted (Permount, Fisher, St Louis), and scored at 400×. Monocytes/macrophages were counted based on their morphology and the characteristic black cytoplasmic granulation Staining for pure and mixed eosinophil colonies was done as described by others[54] using Luxol's Fast Blue and Harris' hematoxylin.

For assay of BFU-E, 1×10^5 low-density or 5×10^4 nonadherent, low-density, T lymphocyte-depleted bone marrow cells were plated, unless otherwise noted, in 35-mm Lux standard tissue culture dishes containing a 1-mL mixture of IMDM, 0.8% methylcellulose, 30% FBS, 5×10^{-5} mol/L of 2 mercaptoethanol, and 1 U of erythropoietin (Hyclone, Logan, UT, or Toyoba, NY) The assay for CFU-GEMM was similar to that for BFU-E except that 0.1 mmol/L of hemin (Eastman Kodak Co, Rochester, NY) was added to these cultures.[55] In some cases, 5637 CM was added as a source of BPA or multi-CSF activities. Dishes were incubated at 37 °C in a humidified atmosphere flushed with 5% CO$_2$ in air or at lowered (5%) O$_2$ tension.[51] Colonies were scored with an inverted microscope after 13 to 15 days of incubation Colonies consisting of more than three erythroid clusters or composed of >500 cells were counted as BFU-E. CFU-GEMM contained granulocytes, erythrocytes, macrophages, and sometimes magakaryocytes. BFU-E-1 colonies were scored on the same plates as CFU-GEMM. Colonies derived from BFU-E-1 were much larger than those derived from BFUE-2, and BFU-E-1 may represent a more immature progenitor than BFU-E-2.

Three plates were scored for each test point, and results are expressed as the mean ±SD. The probability of significant differences between samples was determined with Student's *t* test.

RESULTS

Effect of HuIFNγ on release of activities from PHA-stimulated human T lymphocytes that stimulate GM, erythroid, and mixed colonies. Pure natural HuIFNγ, at concentrations of 0.1, 1, 10, and 100 U/mL, was assessed for its capacity to influence the release of colony-stimulating activities into culture medium conditioned by 10^6 nonadherent low-density, E rosette$^+$ T lymphocytes per milliliter in the presence of PHA for 5 days. The CM was preincubated with double the amount of HuIFNγ neutralizing units (in monoclonal antinatural HuIFNγ) as the amount of HuIFNγ added with the T lymphocytes, prior to assaying the CM on 10^5 low-density normal human bone marrow cells. This was done to prevent the HuIFNγ present in the CM from influencing colony and cluster formation. In the absence of PHA and HuIFNγ, in the presence of HuIFNγ and absence of PHA, and in the absence or presence of monoclonal antinatural HuIFNγ (up to 200 neutralizing units), the T lymphocytes released no detectable colony-stimulating activities for CFU-GM, BFU-E, or CFU-GEMM. CM prepared with cells and PHA, in the absence of HuIFNγ, was preincubated with up to 200 neutralizing units of HuIFNγ, prior to assay Control CM preincubated with monoclonal antinatural HuIFNγ (up to 200 neutralizing units) had the same amount of stimulating activity for GM colony and cluster, erythroid burst, and mixed colony formation as did control CM not preincubated with antibodies (+8% ± 12% nonsignificant change from control CM not preincubated with antibodies). In preliminary experiments, medium conditioned by T lymphocytes in the presence of 1% PHA and in the absence of HuIFNγ (= control) when assayed at 10% vol/vol, stimulated 91 to 217 GM colonies and clusters and 48 to 74 erythroid bursts (ten experiments). In these ten experiments, HuIFNγ enhanced the release from T lymphocytes of activities that stimulated granulocyte and macrophage colonies and clusters (+29% ± 15%, +53% ± 8%, +77% ± 16%, and +79% ± 22%, respectively with 0.1, 1, 10, and 100 U HuIFNγ) but not the release of activities that stimulated erythroid bursts (+5% ± 7%, +1% ± 2%, +2% ± 9%, −4% ± 5%, and +10%, respectively with 0.1, 1, 10, and 100 U of HuIFNγ). When the HuIFNγ-enhancing effects on GM-CSF release were evaluated for stimulation of colonies, the results noted were similar to those for stimulation of colonies plus clusters. In one representative experiment of three, medium conditioned by T lymphocytes in the presence of PHA stimulated 91 ± 7 colonies and 156 ± 16 colonies plus clusters. HuIFNγ, at concentrations of 0.1, 1, and 10 U added to cells with PHA, induced release of GM-CSF that stimulated 127 ± 8 (+39% of control, $P < .05$), 142 ± 3 (+56%, $P < .025$), and 167 ± 7 (+86%, $P < .0025$) colonies, respectively, and 228 ± 9 (+46% of control, $P < .005$), 240 ± 6 (+53%, $P < .005$) and 282 ± 6 (+83%, $P < .00025$) colonies plus clusters. When nonadherent, low-density T lymphocyte-depleted marrows were used instead of low-density marrows as targets for the CM, the results did not change. In three experiments, HuIFNγ at concentrations of 0.1, 1, 10 U enhanced release of GM-CSF, respectively, by 53% ± 4% ($P < .025$), 75% ± 6% ($P < .005$), and 90% ± 3% ($P < .0025$) when nonadherent low-density T lymphocyte target marrows were used These same concentrations of HuIFNγ did not significantly influence release of BPA (+4% ± 3%, −2% ± 2%, and +5% ± 4% of values obtained with CM prepared with PHA in the absence of HuIFNγ).

When T lymphocytes were allowed to condition medium in the presence of either 0.1%, 0.5%, or 1.0% PHA in the absence and presence of 1 and 10 U of natural HuIFNγ, however, it became apparent that HuIFNγ enhanced the release of activities for granulocyte and macrophage colonies and/or clusters, erythroid bursts, and mixed colonies (Tables 1 and 2). HuIFNγ enhanced release of GM colony-stimulating activities from T lymphocytes in the presence of 0.1% to 1.0% PHA; however, the enhancement of the release of burst-promoting and mixed colony activities from T lymphocytes was only detected when these cells were stimulated with 0.1% to 0.5% PHA (Tables 1 and 2). Results were essentially the same whether the CM was prepared in the absence or presence of 10^{-6} mol/L of indomethacin or assessed at 2.5%, 5.0%, or 10.0% vol/vol (Table 2). The enhanced release of GM colony-stimulating activities did not influence the type of the colonies formed (n = two experiments). Thus, T lymphocytes stimulated with 0.1%, 0.5%, or 1.0% PHA in the absence of HuIFNγ stimulated colonies of granulocytes, macrophages, granulocytes plus macrophages (using α-naphthyl acetate esterase plus Mayer's hematoxylin), and pure/mixed eosinophils (using Luxol Fast Blue). The percentage of colony types were, respectively, 31% to 40%, 28% to 40%, 26% to 32%, and 15% to 22%. T lymphocytes in the presence of 0.1% to 1.0% PHA with 10 U of HuIFNγ/mL released activities that stimulated the same percentage of colonies, respectively, in the ranges of 34% to 46%, 25% to 32%, 26% to 35%, and 14% to 28%.

The enhancing effect of HuIFNγ on release of GM-CSF from PHA-stimulated T lymphocytes is due to the HuIFNγ itself, as substantiated by the data in Table 3, which demonstrate that recombinant HuIFNγ mimics the effects of pure natural HuIFNγ (one representative experiment of three is shown) and by the data in Table 4, which demonstrate that monoclonal antinatural HuIFNγ neutralizes the enhancing activity of pure natural HuIFNγ (one representative experiment of three is shown).

Timed addition of HuIFNγ to medium being conditioned by T lymphocytes in the presence of PHA demonstrated that the enhancement of release of GM-CSF was apparent if the HuIFNγ was added at the start of the conditioning medium procedure or after 1 day, but not if the HuIFNγ was added after 2, 3, or 4 days (Table 5).

All the above results were consistent with enhanced

Table 1. Influence of Different Concentrations of HuIFNγ on Release of GM-CSF, Erythroid BPA, and Multi-CSF by T Lymphocytes Stimulated by Varying Concentrations of PHA

Preparation of CM by T Lymphocytes	CFU-GM Colonies	CFU-GM Colonies and Clusters	BFU-E-2 Colonies	BFU-E-1 Colonies	CFU-GEMM Colonies
No CM	0	0	17 ± 5	25 ± 6	0
0.1% PHA + control medium	19 ± 5	66 ± 15	49 ± 4	43 ± 5	1 ± 0
0.1% PHA + 1 U HuIFNγ/mL	42 ± 3(+121)*†	103 ± 8(+56)†	63 ± 4(+29)†	66 ± 9(+53)‡	2 ± 0(+100)†
0.1% PHA + 10 U HuIFNγ/mL	60 ± 6(+216)†	117 ± 10(+77)†	89 ± 2(+82)†	78 ± 2(+81)†	2.5 ± 0 7(+150)†
0 5% PHA + control medium	71 ± 2	88 ± 6	62 ± 8	59 ± 8	3 5 ± 0 7
0.5% PHA + 1 U HuIFNγ/mL	91 ± 8(+28)†	109 ± 4(+24)†	81 ± 3(+31)‡	94 ± 13(+59)†	10 ± 1 4(+186)†
0.5% PHA + 10 U HuIFNγ/mL	100 ± 10(+41)†	136 ± 11(+55)†	92 ± 4(+48)†	82 ± 16(+39)†	11 ± 1 4(+214)†
1.0% PHA + control medium	77 ± 5	117 ± 4	85 ± 5	77 ± 1	10 ± 1.4
1 0% PHA + 1 U HuIFNγ/mL	96 ± 4(+25)†	154 ± 16(+32)†	80 ± 9(−6)	70 ± 1(−9)	11.5 ± 2.1(+15)
1 0% PHA + 10 U HuIFNγ/mL	103 ± 2(+34)†	154 ± 26(+32)‡	82 ± 6(−4)	77 ± 9(0)	9 ± 2 8(−10)

HuIFNγ, human natural interferon γ; GM-CSF, granulocyte-macrophage colony-stimulating factor; BPA, burst-promoting activity; Multi-CSF, mixed colony-stimulating factor; PHA, phytohemagglutinin; CM, conditioned medium; IMDM, Iscove's modified Dulbecco's medium, FBS, fetal bovine serum

One million T lymphocytes per milliliter were incubated in IMDM plus 10% FBS, 10^{-6} mol/L of indomethacin and varying concentrations of PHA in the absence or presence of 1 or 10 U of HuIFNγ. CM was collected after 5 days and incubated with twice the amount of neutralizing units as the amount of HuIFNγ added to cells CM CM was assayed at 10% vol/vol Colonies grown from BFU-E-2 were plated in the presence of 1 U of erythropoietin (Epo)/mL, and colonies grown from BFU-E-1 and CFU-GEMM were plated in the presence of 1 U of Epo plus 0.1 mmol/L of hemin. The results shown are for one of three representative experiments assayed against 4 × 10^4 nonadherent low-density T lymphocyte-depleted bone marrow cells.

*Numbers in parentheses represent the percentage of change from CM prepared in the presence of control (McCoy's) medium.

†Significant percentage of change, $P < .005$

‡Significant percentage of change, $P <$ 05.

release of colony-stimulating activities by PHA-stimulated T lymphocytes in the presence of HuIFNγ. It was also possible that HuIFNγ was suppressing the release from PHA-stimulated T lymphocytes of a suppressor molecule or molecules, however. To evaluate this possibility, medium conditioned by T lymphocytes, in the presence of 0.1%, 0.5%, or 1.0% PHA in the absence of HuIFNγ, or in the presence of 1.0% PHA and 10 U of HuIFNγ, was assessed for its effects on colony formation from marrow CFU-GM stimulated maximally with 10% vol/vol of medium conditioned by the 5637 cell line (Table 6). The T lymphocyte CM had no effect on the 5637 CM stimulation of colony formation (Table 6) or on colony and cluster formation (data not shown). In one experiment, 5637 CM stimulated 145 ± 8 erythroid bursts and 10 ± 2 mixed colonies, and medium conditioned by T lymphocytes in the presence of 1.0% PHA when added with 5637 CM stimulated 154 ± 6 erythroid bursts and 12 ± 3 mixed colonies. These results demonstrate that HuIFNγ was not suppressing the release of suppressor molecules from PHA-stimulated T lymphocytes.

Effect of HuIFNγ on release of colony-stimulating activities from PHA-stimulated subsets of human T lympho-

Table 2. Influence of Conditioned Media Prepared From T Lymphocytes in the Absence and Presence of Varying Concentrations of HuIFNγ and PHA on Colony Formation by CFU-GM, BFU-E, and CFU-GEMM Progenitor Cells

T Lymphocyte-Conditioned Culture Medium in the Presence of		Percentage of Change in Colonies Stimulated by CM Prepared in the Presence of HuIFNγ When CM was Assayed at Different Concentrations							
		CFU-GM			BFU-E			CFU-GEMM	
PHA (%)	HuIFNγ (U)	10% vol/vol	5% vol/vol	2.5% vol/vol	10% vol/vol	5% vol/vol	2 5% vol/vol	10% vol/vol	5% vol/vol
0.1	1	+145 ± 80*	+110 ± 79*	—†	+30 ± 12*	+36 ± 10*	+42 ± 25*	+120 ± 28*	—†
0.1	10	+221 ± 90*	+232 ± 63*	—†	+57 ± 21*	+66 ± 17*	+68 ± 18*	+210 ± 66*	—†
0.5	1	+38 ± 22*	+59 ± 20*	+99 ± 27*	+38 ± 21*	+37 ± 20*	+44 ± 8*	+120 ± 57*	+92 ± 50*
0 5	10	+52 ± 18*	+69 ± 17*	+139 ± 79*	+40 ± 23*	+38 ± 19*	+68 ± 30*	+105 ± 60*	+100 ± 49*
1.0	1	+27 ± 10*	+36 ± 3*	+54 ± 13*	+1 ± 8	+1 ± 10	+14 ± 12	+12 ± 20	−5 ± 7
1 0	10	+47 ± 12*	+60 ± 14*	+74 ± 11*	+8 ± 12	+6 ± 9	+6 ± 6	+8 ± 34	+2 ± 16

Abbreviations as in Table 1

Conditioned medium was prepared as described in the Materials and Methods section prior to assay against 3 × 10^4 to 5 × 10^4 nonadherent low-density T lymphocyte-depleted human bone marrow cells Results are expressed as mean ± SD of the percentage of change from control medium prepared in the absence of HuIFNγ from three different normal donors assayed on a total of three different normal marrows. Each CM was prepared in the absence and presence of 10^{-6} mol/L of indomethacin Results were the same with and without indomethacin, and the data for these were averaged. BFU-E assays were set up with 1 U of erythropoietin ± 0.1 mmol/L of hemin. The results for these cultures were similar and were averaged. Control numbers of colonies for CFU-GM, BFU-E, and CFU-GEMM ranged respectively from 22 to 77, 22 to 94, and 2 to 14 with PHA T lymphocyte CM prepared in the absence of HuIFNγ.

*P at least $<.05$

†Colony numbers too low to evaluate.

Table 3. Comparative Effects of Natural and Recombinant HuIFNγ on Release of GM-CSA From PHA-Stimulated T Lymphocytes

T Lymphocyte-Conditioned Culture Medium in the Presence of PHA and	Release of GM-CSF	
	Colonies ± 1 SD	% Δ*
Control medium	91 ± 10	
0.1 U natural HuIFNγ	127 ± 11	+40†
0 1 U recombinant HuIFNγ	133 ± 11	+46‡
1 U natural HuIFNγ	148 ± 4	+63§
1 U recombinant HuIFNγ	152 ± 9	+67§
10 U natural HuIFN	185 ± 5	+103‖
10 U recombinant HuIFNγ	174 ± 11	+91‖

Abbreviations as in Table 1.

Medium conditioned by 10^6 E rosette$^+$ T lymphocytes was assayed on 10^5 low-density human bone marrow cells as described in Materials and Methods.

*Percentage of change (%Δ) from control medium

†Significant percentage of change, $P < .05$.

‡$P < .005$.

§$P <$ 0025

‖$P < .0005$

Table 4. Monoclonal Anti-HuIFNγ Neutralizes HuIFNγ Enhancement of the Release of GM-CSF From PHA-Stimulated T Lymphocytes

T Lymphocyte-Conditioned Culture Medium in the Presence of PHA and	Release of GM-CSF	
	Colonies and Clusters	%Δ
Control medium	68 ± 5	
10 U HuIFNγ	110 ± 7	+62*
10 U HuIFNγ plus 20 neutralizing U mα HuIFNγ	74 ± 3	+9
20 Neutralizing U mα HuIFNγ	75 ± 4	+10

mα, monoclonal antibody, other abbreviations as in Table 1

Colonies and clusters from 10^5 low-density human bone marrow cells formed in the presence of PHA-stimulated E rosette$^+$ cell-conditioned medium. Control medium McCoy's medium

*Significant percentage of change (%Δ) from control CM, $P < .0025$; other changes are not significant, $P >$ 05.

cytes. Nonadherent, low-density, E rosette$^+$ T lymphocytes were separated by FACS using OKT4 and OKT8 monoclonal antibodies into T4$^+$, T4$^-$, T8$^+$, and T8$^-$ subsets of T lymphocytes. In all experiments, the population of E$^+$ cells was found to contain >98% OKT11$^+$ (or OKT3$^+$) cells, 55% ± 10% OKT4$^+$ and 27% ± 8% OKT8$^+$ cells. After being sorted, the positive fractions contained >98% of cells positive for that marker, whereas the negative cells were contaminated with <1% of the other cell type. In preliminary experiments, the monoclonal antibody dilution (1:100) used to label the E$^+$ cells had no effect on HuIFNγ-induced release of colony-stimulating activities from PHA-stimulated cells. Thus, cells pretreated with control (McCoy's) medium, OKT4 (1:100 dilution) or OKT8 (1:100 dilution) antibodies responded to 1 U of HuIFNγ with significantly enhanced release of GM-CSF with respective values of +47% ± 4%, +47% ± 6%, and +38% ± 7%. Ten units of HuIFNγ caused significant increases in GM-CSF release of 69% ± 4%, 71% ± 5%, and 50% ± 5%, respectively As

Table 5. Release of GM-CSF From PHA-Stimulated T Lymphocytes With Addition of HuIFNγ at Different Time Intervals

T Lymphocyte-Conditioned Culture Medium in the Presence of PHA and	Day of Addition of Control Medium or HuIFNγ	Colonies and Clusters (1 Representative Exp)	(%Δ)* (1 Representative Exp)	%Δ (Average of 3 Exp)
Control medium	0	147 ± 14		
1 U HuIFNγ		209 ± 13	+42†	+45 ± 4‡
10 U HuIFNγ		250 ± 19	+70‡	+78 ± 3§
Control medium	1	151 ± 12		
1 U HuIFNγ		199 ± 15	+32‖	+37 ± 5‡
10 U HuIFNγ		196 ± 8	+30¶	+28 ± 4‖
Control medium	2	171 ± 14		
1 U HuIFNγ		178 ± 22	+4	+5 ± 2
10 U HuIFNγ		181 ± 18	+6	+7 ± 3
Control medium	3	181 ± 18		
1 U HuIFNγ		172 ± 26	−5	−4 ± 3
10 U HuIFNγ		174 ± 16	−4	−7 ± 6
Control medium	4	159 ± 5		
1 U HuIFNγ		165 ± 12	+4	+2 ± 5
10 U HuIFNγ		171 ± 3	+8	+6 ± 4

Abbreviations as in Table 1.

CM was prepared as described in legend to Fig 1, with PHA added on day 0, except that control medium or HuIFN$_{\gamma}$ was added to the cell suspensions on day 0, 1, 2, 3, or 4. The CM was collected on the fifth day of incubation

*Percentage of change (%Δ) from control medium added on the specified day.

†$P <$ 005.

‡$P < .0025$.

§$P < .0005$.

‖Significant %Δ, $P <$ 05.

¶$P < .01$

Table 6. Influence of PHA-Stimulated T Lymphocyte-CM on Colony Formation by CFU-GM Stimulated by 5637 CM

In the Presence of T Lymphocyte CM			Colonies (Day 10 CFU-GM)					
			Exp 1		Exp 2		Exp. 3	
PHA (%)	± 10 U HuIFNγ	± 10^{-6} mol/L Indomethacin	+ Control Medium	+ Anti-HuIFNγ	+ Control Medium	+ Anti-HuIFNγ	+ Control Medium	+ Anti-HuIFNγ
No CM	—	—	62 ± 8	60 ± 3	84 ± 6	—	75 ± 6	—
0.1	–	–	60 ± 14	66 ± 6	—	—	—	—
0.1	–	+	70 ± 1	63 ± 5	—	—	—	—
0.5	–	–	61 ± 3	63 ± 5	—	—	—	—
0.5	–	+	59 ± 6	59 ± 1	—	—	—	—
1.0	–	–	76 ± 7	70 ± 1	—	—	—	—
1.0	–	+	59 ± 2	60 ± 8	—	—	—	—
1.0	–	+	—	—	77 ± 4	83 ± 5	79 ± 3	69 ± 8
1.0	+	+	—	—	80 ± 9	87 ± 11	70 ± 6	81 ± 10

Abbreviations as in Table 1

Medium was conditioned by 10^6 T lymphocytes/mL in the presence of PHA and in the absence and presence of 10 U of natural HuIFNγ/mL and 10^{-6} mol/L of indomethacin. PHA–T lymphocyte CM was then preincubated in the absence and presence of 20 neutralizing U of monoclonal antinatural HuIFNγ for 1½ hours at room temperature and assessed at 10% vol/vol for its effect on colony formation by 4×10^4 nonadherent low-density T lymphocyte-depleted bone marrow cells stimulated by 10% vol/vol 5637 CM PHA–T lymphocyte-CM did not significantly influence colony formation stimulated by 5637 CM

shown in Table 7, 10 U of HuIFNγ/mL significantly enhanced release of activities from $T4^+$ and $T8^-$ cells that stimulated GM colony, erythroid burst, and mixed colony formation, but did not enhance release of these activities from the $T8^+$ or $T4^-$ cells. Similar to the results shown in Tables 1 and 2, enhanced GM activity was released in the presence of 0.1%, 0.5%, and 1.0% PHA, but enhanced release of erythroid burst and mixed colony activities was noted only in the presence of 0.1% and 0.5% PHA (Table 7).

DISCUSSION

Lymphocytes play a role in the regulation of myelopoiesis in vitro[11,12,15–35] and may be involved in myelopoietic regulation in vivo. We have confirmed that T lymphocytes in the

Table 7. Influence of HuIFNγ on Release of GM-CSF, BPA, and Multi-CSF from PHA-Stimulated Subsets of T Lymphocytes Isolated by FACS

T Lymphocyte Subset CM in the Presence of PHA ± 10 U HuIFNγ/mL	CFU-GM	BFU-E	CFU-GEMM
A: Representative experiment expressed as colonies with %Δ from control medium (in parentheses)			
No CM	0	42 ± 1	0
$T4^+$ (0.1% PHA + control medium)	15 ± 2	83 ± 7	0.5 ± 0.7
$T4^+$ (0.1% PHA + HuIFNγ)	26 ± 3 (+73)*	150 ± 2 (+81)*	6 ± 1 (+1,100)*
$T4^+$ (0.5% PHA + control medium)	27 ± 2	88 ± 7	2 ± 1
$T4^+$ (0.5% PHA + HuIFNγ)	48 ± 7 (+78)*	131 ± 13 (+49)*	8 ± 1 (+300)*
$T8^+$ (0.1% PHA + control medium)	17 ± 3	81 ± 10	0.5 ± 0.7
$T8^+$ (0.1% PHA + HuIFNγ)	15 ± 2 (−12)	79 ± 6 (−2)	0.5 ± 0.7 (0)
$T8^+$ (0.5% PHA + control medium)	26 ± 4	80 ± 11	1 ± 1
$T8^+$ (0.5% PHA + HuIFNγ)	27 ± 5 (+4)	73 ± 7 (−9)	1 ± 1 (0)
B: All experiments expressed as %Δ in colonies and clusters stimulated by CM prepared in the presence of HuIFNγ			
$T4^+$ (0.1% PHA)	+81 ± 9*	+96 ± 32†	+800 ± 265†
$T4^+$ (0.5% PHA)	+83 ± 6*	+64 ± 21†	+450 ± 212†
$T4^+$ (1.0% PHA)	+59 ± 22†	+11 ± 10‡	ND
$T8^-$ (1.0% PHA)	+63 ± 14*	+17 ± 15‡	ND
$T8^+$ (0.1% PHA)	−6 ± 8‡	−7 ± 6‡	+5 ± 7
$T8^+$ (0.5% PHA)	+2 ± 3‡	−10 ± 1‡	−4 ± 6
$T8^+$ (1.0% PHA)	+3 ± 9‡	+8 ± 4‡	ND
$T4^-$ (1.0% PHA)	+18 ± 16‡	+19 ± 15‡	ND

CM from 0.5 to 1.0 × 10^6 T cell subsets was prepared as described in the Materials and Methods section and incubated prior to assay with 20 neutralizing U of monoclonal HuIFNγ prior to assay against 3 to 5 × 10^4 nonadherent low-density T lymphocyte-depleted bone marrow cells Results are expressed as mean ± SD. For part B, results for CM prepared with 0.1% and 0.5% PHA were from two sorting experiments, each assayed on two different marrows; results for CM prepared with 1.0% PHA were from four sorting experiments assayed on a total of four different marrows Control numbers of CFU-GM colonies and clusters, BFU-E colonies, and CFU-GEMM colonies ranged from 20 to 127, 47 to 88, and 0.5 to 2, respectively, with phytohemagglutinin (PHA) T lymphocyte subset-stimulated CM prepared in the absence of HuIFNγ.

*Significant %Δ from control, $P < .0025$.

†Significant %Δ from control, $P < .005$.

‡Not significant, $P > .05$.

absence of other cell types, or in the presence of very small numbers of other cell types, do not release GM-CSF, BPA, or multi-CSF activities unless the T lymphocytes are stimulated by an agent such as PHA. Both the T4 antigen$^+$ and T8 antigen$^+$ lymphocyte subsets released these activities in the presence of PHA.

Many functions have been identified for HuIFNγ in vitro; to this list is now added the enhancement of release of GM-CSF, BPA, and multi-CSF activities from T4 antigen$^+$, but not from T8 antigen$^+$, lymphocyte subsets. That it is HuIFNγ itself that is enhancing release of these activities is substantiated by the use of pure natural HuIFNγ, neutralization of the GM-CSF enhancing effects of pure natural HuIFNγ with a monoclonal antibody that recognizes natural HuIFNγ, and duplication of this enhancing effect with purified recombinant HuIFNγ. The HuIFNγ was not suppressing the release of suppressor molecules from PHA-stimulated T lymphocytes, but our results do not rule out the possibility that the HuIFNγ effects are being mediated by another messenger released in response to HuIFNγ. Such a possibility has been shown by others for the induction of Ia antigens on myeloid cells by IFNγ.[56]

Use of T lymphocyte- and monocyte-depleted bone marrow cells to assess the stimulatory capacity of blood T lymphocyte CM suggests that it is a stimulating rather than a potentiating activity for CFU-GM and CFU-GEMM that is being released from PHA-stimulated T lymphocytes in the absence and presence of HuIFNγ. None of the colony-stimulating activities were released in response to HuIFNγ in the absence of PHA, suggesting that HuIFNγ alone is not capable of inducing release of GM-CSF, BPA, or multi-CSF. Induction of GM-CSF and BPA from lymphocytes is sometimes but not always concordant with respect to production and kinetics of release.[29–32,34] It is not possible from our data to conclude whether the enhanced release of activities for GM colonies, erythroid bursts, and mixed colonies are due to a single molecule, or to several factors. Pure recombinant human GM-CSF[57–59] and G-CSF[60] can stimulate multilineages of human cells in vitro.

The loss of responsiveness of T lymphocytes with time in culture to the enhancing effects of HuIFNγ could relate to loss of receptors for HuIFNγ and/or to loss of antigenic determinants necessary for its binding and/or activity. Alternatively, the inability to detect the enhancing effect of HuIFNγ after a few days in culture may only reflect endogenously released HuIFNγ, which may be acting on the cells and masking the effect of exogenously added HuIFNγ.

Whether the function for HuIFNγ described in this article or functions described elsewhere for HuIFNγ in vitro are meaningful in terms of the regulation of myelopoiesis in vivo remains to be determined. Demonstrating relevance in vivo for a function noted in vitro is difficult, and the difficulty is compounded when many complex cell–cell or molecule–cell interactions are possible that can modify or counterbalance an effect HuIFNγ may act potentially as a positive stimulus for myelopoiesis in vivo by enhancing release of CSF from activated T4 antigen$^+$ lymphocytes, by enhancing release of GM-CSF[10,48,49] from MHC class II antigen$^+$ monocytes,[10] and by decreasing release from monocytes of acidic isoferritins[57] that suppress hematopoietic progenitor cells.[52,58] HuIFNγ may also act potentially as a negative stimulus for myelopoiesis in vivo, however, by suppressing hematopoietic progenitor cells directly or indirectly.[38–40] Moreover, HuIFNγ can induce MHC class II antigens on monocytes[42–44] and CFU-GM[45] in vitro, and induction of increasing density expression of MHC class II antigens on a monocyte cell line[46,47] and CFU-GM[63] has been associated with induction of increased sensitivity of these cell populations to suppression by lactoferrin and acidic isoferritins, respectively. The result in vivo may depend on time, and cell-type, and molecule concentration. This may actually involve a sequence of events in which low concentrations of HuIFNγ induce a positive effect that can in time be limited by HuIFNγ induction of responsiveness of cells to the action of suppressor molecules other than HuIFNγ and, eventually, by direct HuIFNγ suppression due to increasing concentrations of HuIFNγ itself.

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REFERENCES

1 Broxmeyer HE: Hematopoietic stem cells, in Trubowitz S, Davis S (eds): The Human Bone Marrow. Boca Raton, FL, CRC Press, 1982, p 77

2 Broxmeyer HE Granulopoiesis, in Trubowitz S, Davis S (eds): The Human Bone Marrow. Boca Raton, FL, CRC Press, 1982, p 145

3. Metcalf D. The granulocyte-macrophage colony stimulating factors. Science 229.16, 1985

4. Metcalf D. The granulocyte-macrophage colony stimulating factors Cell 43:5, 1985

5. Moore MAS, Williams N. Physical separation of colony-stimulating cells from in vitro colony forming cells in hemopoietic tissue. J Cell Physiol 80:195, 1972

6. Chervenick PA, Lobuglio AF Human blood monocytes· Stimulators of granulocyte and mononuclear colony formation in vitro. Science 178:164, 1972

7. Golde DW, Finley TN, Cline MJ: Production of colony stimulating factor by human macrophages. Lancet 2:1397, 1972

8. Broxmeyer HE, Platzer E: Lactoferrin acts on I-A and I-E/C antigen-positive subpopulations of mouse peritoneal macrophages in the absence of T-lymphocytes and other cell types to inhibit production of granulocyte-macrophage colony stimulating factors in vitro J Immunol 133:306, 1984

9. Sullivan R, Gans PJ, McCarroll LA: The synthesis and secretion of granulocyte-monocyte colony stimulating activity (CSA) by isolated human monocytes: Kinetics of the response to bacterial endotoxin. J Immunol 130 800, 1983

10. Piacibello W, Lu L, Wachter M, Rubin BY, Broxmeyer HE: Release of granulocyte-macrophage colony stimulating factors from major histocompatibility complex class II antigen-positive monocytes is enhanced by human gamma interferon Blood 66·1343, 1985

11 Verma DS, Spitzer G, Zander AR, Fisher R, McCredie KB, Dicke KA: T lymphocyte and monocyte macrophage interaction in colony-stimulating activity elaboration in man. Blood 54:1376, 1979

12. Bagby GC Jr, Regas VD, Bennett RM, Vandenbark AA, Garewal HS· Interaction of lactoferrin, monocytes and T-lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. J Clin Invest 68:56, 1981

13. Bagby GC Jr, McCall E, Bergstrom KA, Burger D: A monokine regulates colony-stimulating activity production by vascular endothelial cells Blood 62:663, 1983

14. Bagby GC Jr, McCall E, Layman DL: Regulation of colony-stimulating activity production. Interactions of fibroblasts, mononuclear phagocytes and lactoferrin. J Clin Invest 71:340, 1983

15. Nathan DG, Chess L, Hillman DG, Clarke B, Beard J, Merler E, Housman DE. Human erythroid burst-forming unit: T-cell requirement for proliferation *in vitro* J Exp Med 147 324, 1978

16. Torok-Storb B, Martin PJ, Hansen JA Regulation of *in vitro* erythropoiesis by normal T cells: Evidence for two T-cell subsets with opposing function. Blood 58.171, 1981

17. Kurland JI, Meyers PA, Moore MAS· Synthesis and release of erythroid colony- and burst-potentiating activities by purified populations of murine peritoneal macrophages. J Exp Med 151:839, 1980

18. Zuckerman KS: Stimulation of human BFU-E by products of human monocytes and lymphocytes Exp Hematol 8.924, 1980

19. Mangan KF, Desforges JF The role of T lymphocytes and monocytes in the regulation of human erythropoietic peripheral blood burst forming units. Exp Hematol 8.717, 1980

20. Zuckerman KS· Human erythroid burst-forming units, Growth in vitro is dependent on monocytes, but not T lymphocytes J Clin Invest 67 702, 1981

21. Reid CDL, Baptista LC, Chanarin I: Erythroid colony growth *in vitro* from human peripheral blood null cells: Evidence for regulation by T-lymphocytes and monocytes. Br J Haematol 48:155, 1981

22. Linch DC, Nathan DG: T cell and monocyte derived burst-promoting activity directly act on erythroid progenitor cells. Nature 312:775, 1984

23. Linch DC, Lipton JM, Nathan DG. Identification of three accessory cell populations in human bone marrow with erythroid burst-promoting activity. J Clin Invest 75:1278, 1985

24. Levitt L, Kipps TJ, Engleman EG, Greenberg PL. Human bone marrow and peripheral blood T lymphocyte depletion: Efficacy and effects of both T cells and monocytes on growth of hematopoietic progenitors. Blood 65:663, 1985

25 Dainiak N, Feldman L, Cohen CM: Neutralization of erythroid burst-promoting activity *in vitro* with antimembrane antibodies. Blood 65.877, 1985

26. Cline MJ, Golde DW Production of colony-stimulating activity by human lymphocytes. Nature 248:703, 1974

27. Ruscetti FW, Chervenick PA: Regulation of the release of colony stimulating activity from mitogen-stimulated lymphocytes. J Immunol 114:1513, 1975

28 Broxmeyer HE, Lu L, Bognacki J· Transferrin, derived from an OKT8-positive subpopulation of T-lymphocytes, suppresses the production of granulocyte-macrophage colony stimulatory factors from mitogen-activated T-lymphocytes Blood 62.37, 1983

29 Griffin JD, Meuer SC, Schlossman SF, Reinherz EL. T cell regulation of myelopoiesis. Analysis at a clonal level. J Immunol 133.1863, 1984

30 Staber FG, Hultner L, Marcucci F, Krammer PH Production of colony stimulating factors by murine T cells in limiting dilution and long-term cultures Nature 298:79, 1982

31. Kelso A, Metcalf D· Clonal heterogeneity in colony stimulating factor production by murine T lymphocytes. J Cell Physiol 123:101, 1985

32. Pantaleo G, Moretta A, Cillo C, Eliason JF, Moretta L: Phenotypic and functional heterogeneity of human peripheral blood T lymphocytes producing colony stimulatory factor. A clonal and precursor frequency analysis. J Immunol 135:3933, 1985

33 Hesketh PJ, Sullivan R, Valeri CR, McCarroll LA The production of granulocyte-monocyte colony-stimulating activity by isolated human T lymphocyte subpopulations. Blood 63:1141, 1984

34. Sullivan R, Hesketh PJ, Zuckerman KS: Kinetics of the production of granulocyte-monocyte colony stimulating activity (GM-CSA) and erythroid burst promoting activity (BPA) by human T-lymphocyte subsets. Blood 64:454a, 1984 (suppl 1) (abstr)

35. Wisniewski D, Strife A, Wachter M, Clarkson B· Regulation of human peripheral blood erythroid burst-forming unit growth by T lymphocytes and T lymphocyte subpopulations defined by OKT4 and OKT8 monoclonal antibodies. Blood 65·456, 1985

36. Stewart WE II The Interferon System New York, Springer-Verlag, 1979

37. Pestka S, Langer JA, Fisher PB, Weinstein IB, Ortaldo J, Herberman RB. The human interferons: From the past into the future, in Ford RJ, Maizel AL (eds)· Mediators in Cell Growth and Differentiation New York, Raven Press, 1985, p 261

38. Broxmeyer HE, Lu L, Platzer E, Feit C, Juliano L, Rubin BY: Comparative analysis of the influences of human gamma, alpha and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells J Immunol 131:1300, 1983

39. Broxmeyer HE, Cooper S, Rubin BY, Taylor MW: The synergistic influence of human interferon-γ and interferon-α on suppression of hematopoietic progenitor cells is additive with the enhanced sensitivity of these cells to inhibition by interferons at low oxygen tension *in vitro* J Immunol 135·2502, 1985

40. Raefsky EL, Platanias LC, Zoumbos NC, Young NS. Studies of interferon as a regulator of hematopoietic cell proliferation J Immunol 135:2513, 1985

41. Rubin BY, Bartal AH, Anderson SL, Millet SK, Hershaut Y, Feit C· The anticellular and protein inducing activities of human interferon preparations are mediated by the interferon. J Immunol 130:1019, 1983

42. Wallach D, Fellow M, Revel M: Preferential effect of interferon on the synthesis of HLA antigens and their mRNAs in human cells. Nature 299:833, 1982

43. King DR, Jones PP· Induction of Ia and H-2 antigens on a macrophage cell line by immune interferon. J Immunol 131:315, 1983

44 Steeg PS, Moore RN, Johnson HM, Oppenheim JJ: Regulation of murine macrophage Ia antigen expression by a lymphokine with immune interferon activity. J Exp Med 156:1780, 1982

45. Griffin JD, Sabbath KD, Herrman F, Larcom P, Nichols K, Kornacki M, Levine H, Cannistra SA: Differential expression of HLA-DR antigens in subsets of human CFU-GM. Blood 66·788, 1985

46. Broxmeyer HE, Piacibello W, Juliano L, Platzer E, Berman E, Rubin BY: Gamma interferon induces colony forming cells of the human monoblast cell line U937 to respond to inhibition by lactoferrin, transferrin and acidic isoferritins Exp Hematol 14:35, 1986

47. Piacibello W, Rubin BY, Broxmeyer HE. Prostaglandin E counteracts modulation of the gamma interferon induction of major histocompatibility complex class II antigens on U937 cells and induction of responsiveness of U937 colony forming cells to suppression by lactoferrin, transferrin, acidic isoferritins and prostaglandin E. Exp Hematol 14 44, 1986

48. Schlick E, Hartung K, Stevenson HC, Chirigos MA: Secretion of colony-stimulating factors by human monocytes and bone marrow cells after in vitro treatment with biological response modifiers J Leuk Biol 37:615, 1985

49. Herrman F, Cannistra SA, Griffin JD: T cell-monocyte interactions in the production of humoral factors regulating human granulopoiesis in vitro. J Immunol 136:2856, 1986

50. Pelus LM, Broxmeyer HE, Kurland JI, Moore MAS: Regulation of macrophage and granulocyte proliferation: Specificities of prostaglandin E and lactoferrin. J Exp Med 150:277, 1979

51 Lu L, Broxmeyer HE Comparative influences of phytohemagglutinin-stimulated leukocyte conditioned medium, hemin, prostaglandin E, and low oxygen tension on colony formation by erythroid progenitor cells in normal human bone marrow. Exp Hematol 13:989, 1985

52. Broxmeyer HE, Bognacki P, Ralph P, Dorner MH, Lu L, Castro-Malaspina H. Monocyte-macrophage derived acidic isoferritins: normal feedback regulators of granulocyte-macrophage progenitor cells Blood 60:595, 1982

53. Jacobsen N, Broxmeyer HE, Grossbard E, Moore MAS: Colony forming units in diffusion chambers (CFU-D) and colony forming units in agar culture (CFU-C) obtained from human bone marrow A possible parent progeny relationship. Cell Tissue Kinet 12:213, 1979

54. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Preparation of colony-stimulating factors from human placental conditioned medium. Leuk Res 2:313, 1978

55. Lu L, Broxmeyer HE· The selective influence of hemin and products of erythrocytes on colony formation of human erythroid (BFU-E) and multipotential (CFU-GEMM) progenitor cells *in vitro* Exp Hematol 11:721, 1983

56. Walker E, Maino V, Sanchez-Lanier M, Warner N, Stewart C: Murine Gamma interferon activates the release of a macrophage-derived-Ia-inducing factor that transfers Ia inductive capacity. J Exp Med 159:1532, 1984

57. Emerson SG, Sieff CA, Wang EA, Wong GG, Clark SC, Nathan DG: Purification of fetal hematopoietic progenitors and demonstration of recombinant multipotential colony-stimulating activity. J Clin Invest 76:1286, 1985

58. Sieff CA, Emerson SG, Donahue RE, Nathan DG, Wang EA, Wong GG, Clark SC: Human recombinant granulocyte-macrophage colony stimulating factor: A multilineage hematopoietin. Science 230·1171, 1985

59. Kaushansky K, O'Hara PJ, Berkner K, Segal GM, Hagen FS, Adamson JW: Genomic cloning, characterization and multilineage growth-promoting activity of human granulocyte-macrophage colony-stimulating factor. Proc Natl Acad Sci USA 83:3101, 1986

60. Souza LM, Boone TC, Gabrilove J, Lai PH, Zsebo KM, Murdock DC, Chazin VR, Bruszewski J, Lu H, Chen KK, Barendt J, Platzer E, Moore MAS, Mertelsmann R, Welte K: Recombinant human granulocyte colony-stimulating factor: Effects on normal and leukemic cells. Science 232:61, 1986

61. Broxmeyer HE, Rubin BY, Juliano L, Platzer E, Lu L: The HLA-DR restricted action of T-lymphocyte subsets on modulation of release of acidic isoferritin-inhibitory activity from monocytes is mediated by alpha and gamma interferon, interleukin 1, and transferrin. Blood 62.131a, 1983 (suppl 1) (abstr)

62. Lu L, Broxmeyer HE, Meyers PA, Moore MAS, Thaler HT: Association of cell cycle expression of Ia-like antigenic determinants on normal human multipotential (CFU-GEMM) and erythroid (BFU-E) progenitor cells with regulation *in vitro* by acidic isoferritins. Blood 61.250, 1983

63. Broxmeyer HE: Relationship of cell-cycle expression of Ia-like antigenic determinants on normal and leukemia human granulocyte-macrophage progenitor cells to regulation *in vitro* by acidic isoferritins. J Clin Invest 69:632, 1982

Stromal Cells in Myeloid and Lymphoid Long-Term Bone Marrow Cultures Can Support Multiple Hemopoietic Lineages and Modulate Their Production of Hemopoietic Growth Factors

By Anne Johnson and Kenneth Dorshkind

Hemopoiesis in long-term bone marrow cultures (LTBMC) is dependent on adherent stromal cells that form an in vitro hemopoietic microenvironment. Myeloid bone marrow cultures (MBMC) are optimal for myelopoiesis, while lymphoid bone marrow cultures (LBMC) only support B lymphopoiesis. The experiments reported here have made a comparative analysis of the two cultures to determine whether the stromal cells that establish in vitro are restricted to the support of myelopoiesis or lymphopoiesis, respectively, and to examine how the different culture conditions affect stromal cell physiology. In order to facilitate this analysis, purified populations of MBMC and LBMC stroma were prepared by treating the LTBMC with the antibiotic mycophenolic acid; this results in the elimination of hemopoietic cells while retaining purified populations of functional stroma. Stromal cell cultures prepared and maintained under MBMC conditions secreted myeloid growth factors that stimulated the growth of granulocyte-macrophage colonies, while no such activity was detected from purified LBMC stromal cultures. However, this was not due to the inability of LBMC stroma to mediate this function. Transfer of LBMC stromal cultures to MBMC conditions resulted in an induction of myeloid growth factor secretion. When seeded under these conditions with stromal cell-depleted populations of hemopoietic cells, obtained by passing marrow through nylon wool columns, the LBMC stromal cells could support long-term myelopoiesis. Conversely, transfer of MBMC stroma to LBMC conditions resulted in a cessation of myeloid growth factor secretion; on seeding these cultures with nylon wool-passed marrow, B lymphopoiesis, but not myelopoiesis, initiated. These findings indicate that the stroma in the different LTBMC are not restricted in their hemopoietic support capacity but are sensitive to culture conditions in a manner that may affect the type of microenvironment formed.

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RESULTS from a number of experimental studies have suggested that the development of various hemopoietic lineages and maturational states in the bone marrow does not occur randomly in the medullary cavity, but is localized in distinct microenvironments.[1-3] Considerable attention has focused on a population of supporting elements known as stromal cells in the formation of these niches.[4] These are a presumably nonhemopoietic population of cells on which active blood cell formation is dependent (reviewed in reference 5). The precise means by which stromal cells could form a particular microenvironment is unknown. One possibility is that subpopulations of stromal cells exist that specifically support a particular lineage or maturational state. This would necessitate that considerable stromal cell heterogeneity exists Morphological examination of in situ bone marrow has identified a variety of stromal cell types that include reticular cells, endothelial cells, fibroblasts, and fat cells,[6,7] and in vitro analysis of stromal cell clones has provided additional evidence of heterogeneity by describing various subpopulations based on morphology or extracellular matrix secretion.[8,9] Whether or not all these populations are functional in vivo is unknown. Alternatively, the number of functional stromal cell populations that exist may be limited, and one stromal cell type may be able to support multiple hemopoietic lineages and maturational states.

The analysis of different types of long-term bone marrow cultures (LTBMC) offers an in vitro approach to investigate the formation of hemopoietic microenvironments. The common feature of LTBMC is the establishment in vitro of an adherent layer of stromal cells on which long-term hemopoiesis is dependent.[10] Considerable variation in conditions used to establish the cultures exists, and this results in significant differences in the pattern of hemopoiesis observed. The myeloid bone marrow cultures (MBMC) described by Dexter et al produce spleen colony-forming units (CFU-S) and differentiated myeloid cells and their progenitors.[11] Mature lymphocytes are not detected in the cultures, but a B cell precursor is present[12-15] that has been shown to be a primitive hemopoietic cell.[16] The lymphoid bone marrow cultures (LBMC) described by Whitlock and Witte[17] are optimal for B lymphopoiesis and not myelopoiesis. Mature B cells and their precursors are detectable in these LBMC for up to several months, while CFU-S, myeloid progenitors, and mature myeloid cells are not detected by the first few weeks after culture initiation.[18-20]

The culture conditions under which these LTBMC are established undoubtedly influence the pattern of hemopoiesis by direct action on the hemopoietic cells themselves.[21] It is also possible that distinct hemopoietic microenvironments are formed in the LTBMC as a result of the culture conditions. This could be a result of the selective growth of stromal cell subpopulations that only support myelopoiesis or lymphopoiesis. A nonmutually exclusive alternative is that the different culture conditions used affect the means by which the stromal cells function and the type of microenvironment formed. The purpose of this investigation is to distinguish between these alternatives. The findings indicate that stromal cells in the respective LTBMC are not restricted to the support of myelopoiesis and lymphopoiesis. However,

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the culture conditions used affect stromal cell function, and this appears to contribute to the type of microenvironment that is created.

MATERIALS AND METHODS

Mice BALB/cAn mice were obtained from Life Sciences, St Petersburg, FL, or bred in the vivarium of the Division of Biomedical Sciences, University of California, Riverside. Severe combined immunodeficient (SCID) mice were bred and maintained under sterile, laminar flow conditions in the divisional vivarium from homozygous breeding stock obtained from the Ontario Cancer Institute, Canada (a gift of Drs RA Phillips and MJ Bosma). The genetics of this mutant and its characterization have been previously described [22] Male or female mice were used at 4 to 6 weeks of age. Briefly, SCID mice have an autosomal recessive defect that results in the absence of lymphocytes in their hemopoietic tissues. However, the microenvironment in these mutants is normal and lymphocytes can be reconstituted without prior irradiation with grafts of normal stem cells [23]

Preparation of cell suspensions. Animals were sacrificed by cervical dislocation, and spleens, femurs, and tibiae removed and placed in α-minimal essential medium (α-MEM; GIBCO, Grand Island, NY) Bone marrow cells were flushed from the bones with a syringe and needle and single-cell suspensions prepared by gentle pipetting. Cell viability was determined by eosine dye exclusion, and all cell counts were performed in a hemocytometer.

Preparation of mycophenolic acid. Mycophenolic acid (MPA), a gift of the Eli Lilly Co, Indianapolis, was prepared by dissolving 10 mg of the drug in 400 μL of 95% ethanol at room temperature. Once in solution, a 1-mg/mL stock was obtained by adding phosphate-buffered saline to a final volume of 10 mL The solution was sterile filtered and stored at 4 °C. Fresh stock solutions were prepared every 3 weeks, as loss of activity was noted with older preparations.

Preparation of stromal cell cultures Stromal cell cultures were generated by establishing long-term bone marrow cultures under myeloid or lymphoid conditions and subsequently treating these with MPA [24] Cultures were initiated with marrow from either BALB/cAN or SCID mice The stromal cells in these latter mice are normal, and no differences were observed between experiments using SCID or BALB/c stromal cells. MBMC were established by flushing the contents of one femur and tibia into a 25-cm^2 tissue culture flask (#3013, Falcon, Oxnard, CA) containing 8 mL of α-MEM supplemented with 20% horse serum (GIBCO) and 10^{-6} mol/L hydrocortisone sodium succinate (Upjohn, Kalamazoo, MI). The cultures were then gassed with a 5% CO_2, 95% air mixture, tightly sealed, and placed in a dry 33 °C incubator. Cultures were fed after 1 week, and by 2 weeks a confluent, adherent layer of cells had been established. MBMC are usually recharged at this time by adding fresh bone marrow cells to the cultures. Instead, MBMC were treated with MPA in order to prepare purified stromal cell layers. All culture medium was removed from the flasks and replaced with α-MEM supplemented with 20% horse serum, 10^{-6} mol/L hydrocortisone, and 5 μg/mL MPA. Three days later all the MPA-containing medium was replaced with fresh medium After an additional three-day incubation, the cycle was repeated. In most cases, two cycles of drug treatment were sufficient to eliminate hemopoietic cells. It has been shown that no hemopoietic colonies can be detected among the cells harvested from MPA-treated cultures, indicating that the procedure does eliminate hemopoietic cells.[24] All cultures were maintained at least 2 weeks after the last drug treatment before use.

LBMC were established by plating 13 5 mL of a single-cell suspension of bone marrow cells at 10^6 cells per milliliter in RPMI-1640 medium supplemented with 5% fetal calf serum (GIBCO) and 5×10^{-5} mol/L 2-mercaptoethanol (2-ME, Sigma Chemical Co, St Louis) in 10-cm^2 tissue culture dishes (Corning Plastics, Santa Clara, CA). Cultures were fed twice a week as described previously.[17] At the end of 2 weeks, a confluent, adherent layer had established and cultures were treated with MPA according to the protocol noted earlier.

In some experiments, adherent cells were harvested from LTBMC by removing all the culture medium and replacing it with 2 mL of a 0 15% collagenase solution in phosphate-buffered saline supplemented with 1 μg/mL DNAse (Sigma). After incubation for 15 minutes at 37°C, adherent cells were gently removed by scraping with a rubber policeman. Viability of the harvested cells was always 95% to 100%

Separation of cells on nylon wool column. Cells from fresh bone marrow were passed through nylon wool columns prepared according to the method of Julius et al [25] in order to deplete stromal cells. A maximum of 5×10^7 fresh bone marrow cells in a 2-mL volume was loaded onto a column packed in a 12-mL syringe and washed into the nylon wool with 1 mL of 37°C medium. The nonadherent cells were eluted from the column by washing with 20 mL of the medium. The eluted cells were then passed through a second column as described earlier. Cells were incubated for 45 minutes on each column. Approximately 7% of the initial cell input remained after passage through the second nylon wool column. To confirm the efficacy of the separation procedure, aliquots of 5×10^5 of the twice-nylon wool-passed cells were cultured alone in the appropriate medium and observed throughout the course of the experiment for growth of stromal or hemopoietic populations

B lymphocyte colony-forming assay. Colony-forming B cells (CFU-B) were assayed as described by Kincade et al [26] Nine parts of Iscove's modified Dulbecco's medium supplemented with 15% fetal calf serum, 0.4% nonessential amino acids, 0 8% essential amino acids, 0.4% vitamin mixture (all from GIBCO), 0.6% $NaHCO_3$, and 5×10^{-5} mol/L 2-ME were mixed with one part 3% agar (Difco, Detroit) dissolved by boiling in double distilled H_2O. In addition, the medium contained 0.05% sheep red blood cells (Colorado Serum, Denver) that had been washed four times before use and 10 μg/mL *Escherichia coli* lipopolysaccharide (LPS, Difco) The medium was mixed with 1×10^5 cells in 0.2 mL and plated in 1-mL volumes in 35-mm plastic Petri dishes (Falcon) After the agar had gelled, cultures were placed in a humidified 5% CO_2 and 95% air incubator at 37 °C After six days of incubation, red cells were lysed by adding 1 mL of 3 0% glacial acetic acid in distilled H_2O to allow visualization of B cell colonies

Antibody labeling of cells and fluorescence microscopy Surface IgM-bearing cells were identified with an affinity-purified, fluorescein-conjugated goat antimouse IgM (Southern Biotechnology, Birmingham, AL) This reagent was diluted 1:5 in α-MEM supplemented with 5% fetal calf serum and 1 mg/mL sodium azide Aliquots of 1 to 2×10^6 cells harvested from cultures or fresh bone marrow were suspended in 100 μL of the diluted antiserum and incubated for 45 minutes on ice. Cells were then washed twice at 4 °C in α-MEM without serum and resuspended in 0 2 mL of α-MEM To this was added an equivalent volume of 2% paraformaldehyde in phosphate-buffered saline Cells were stored at 4 °C until cell counts were performed on wet mount preparations using a E. Leitz (Rockleigh, NJ) Laborlux microscope equipped for epifluorescence. At least 500 cells were counted per sample.

Myeloid colony assay. Myeloid progenitor cells capable of forming colonies in semisolid medium were assayed by culturing 5×10^4 cells in 35-mm plastic Petri dishes containing 1 mL of methylcellulose medium.[27] The latter contained 0.8% methylcellulose, 30% fetal calf serum, 5×10^{-5} mol/L 2-ME, and 10% medium conditioned by WEHI-3B (D^-) cells. This was prepared by culturing WEHI cells at 10^6 cells per milliliter in 40 mL of α-MEM supplemented with 1% fetal calf serum. After a 24-hour incubation, supernatants were collected, centrifuged, and stored at 4°C until used. Colonies were enumerated on day 8.

Collection of conditioned medium Medium conditioned by MPA-treated stromal cell cultures was harvested and tested for the presence of colony-stimulating factors in some experiments On day 0 all medium was removed from the cultures and replaced with either fresh α-MEM supplemented with 20% horse serum and 10^{-6} mol/L steroids or RPMI-1640 supplemented with 5% fetal calf serum and 5×10^{-5} mol/L 2-ME. At various time points thereafter, medium from three replicate flasks was collected and pooled. A separate set of cultures was used for collection of medium at different time points. Cultures initiated under MBMC or LBMC conditions were switched to LBMC or MBMC conditions, respectively, in some experiments, and conditioned medium was collected as noted. Cultures were allowed to adapt to the new culture conditions for 2 weeks before collection of medium.

RESULTS

Effects of culture conditions on stromal cells. Investigations on stromal cell lines have demonstrated that these cells are sensitive to the culture conditions under which they are maintained.[28] This suggests that the different culture conditions in MBMC and LBMC might also affect stromal cells, and this in turn could influence hemopoiesis in the respective cultures.

As a simple, reproducible assay of stromal cell function, the ability of stromal cells in the LTBMC to condition medium with growth factors that could stimulate the development of granulocyte-macrophage progenitors was used. Because hemopoietic cells in intact cultures could possibly bind to the growth factors and decrease their concentration in the supernatants, the analysis was conducted using purified MBMC and LBMC stromal cell cultures. This was accomplished by treating the cultures with MPA as described in Materials and Methods. The data in Table 1 indicate that there are differences with regard to growth factor secretion by MPA-treated MBMC and LBMC stromal cell cultures. Myeloid colony-stimulating activity was present in supernatants conditioned by MBMC stroma, with the highest activity at one day after feeding of the cultures. Minimal activity was present in supernatants conditioned by LBMC stroma. However, this was not due to an inability of LBMC stromal cells to produce this substance; after transfer to MBMC conditions, stromal cells initiated under LBMC conditions could secrete myeloid growth factors. Conversely, a marked decline in their production by MBMC stroma was observed after transfer to LBMC conditions. Minimal CFU-GM growth was stimulated by horse or fetal calf serum used to grow the cultures (Table 1 footnote). The molecular species of the secreted colony-stimulating factors has not been determined.

The photomicrographs in Fig 1 demonstrate that stromal cell morphology is also affected by the culture conditions. MPA-treated stroma was used to eliminate hemopoietic cells that might obscure the stroma. Fat cells are a prominent feature of the adherent layer in MBMC (Fig 1A), but these disappear almost entirely after transfer to LBMC conditions (Fig 1B). Fat cells are not readily observed in LBMC (Fig 1C); after transfer to MBMC conditions, these appeared and the stroma took on an appearance characteristic of a primary MBMC (Fig 1D).

LBMC stroma can support myelopoiesis after transfer to MBMC conditions. Because LBMC stromal cells were capable of myeloid growth factor production, it was possible that they could support long-term myelopoiesis in vitro. This was tested by treating LBMC with MPA, seeding the cultures with 5×10^5 nylon wool-passed marrow cells in MBMC medium, and transferring them to 33 °C. The nylon wool passage removes stromal cells from the marrow that can form an adherent stromal cell layer in vitro, thus ensuring that precursors in the reseed population are dependent for growth on the preestablished LBMC stroma.[24] The addition of nylon wool-passed hematopoietic cells to LBMC stroma was essential in order to supply a source of myeloid precursors, since the latter are not detectable in LBMC.[18-20,29] The rationale for using MPA-treated stroma was to eliminate any

Table 1. Culture Conditions Affect Production of Colony-Stimulating Factors by Stromal Cells

Day Supernatant Collected	No of CFU-GM/5 × 10^4 Stimulated by Supernatants From			
	MBMC	MCMB → LBMC	LBMC	LBMC → MBMC
Experiment 1*				
1	91 ± 13	7 ± 7	1	60 ± 13
4	41 ± 2	4 ± 2	.5 ± 7	49 ± 11
7	36 ± 4	5 ± .7	1 ± 1 4	32 ± 21
Experiment 2†				
3	59 ± 8	1 ± 1.4	0	11 ± 1
Experiment 3				
1	100 ± 0	5 ± 4	ND	ND

Supernatants were collected from primary cultures 2 weeks after the last MPA treatment. Cultures transferred to alternate conditions were allowed to adapt to the different medium for 2 weeks before collection of supernatants Values are expressed as mean ± SD.

Abbreviation: ND, not determined

*Frequency of CFU-GM/5 × 10^4 BALB/c bone marrow cells stimulated by WEHI-3 conditioned medium was 63 ± 10 A 10% mixture of culture medium containing 5% fetal calf serum or 20% horse serum only as stimulator instead of WEHI-conditioned medium stimulated the growth of 0 5 ± 0 7 and 1.5 ± 0 7 CFU-GM/5 × 10^4 marrow cells, respectively, in methylcellulose cultures.

†Frequency of CFU-GM/5 × 10^4 BALB/c bone marrow cells stimulated by WEHI-3-conditioned medium was 32 ± 5. A 10% mixture of culture medium containing 5% fetal calf serum or 20% horse serum only as stimulator instead of WEHI-conditioned medium stimulated the growth of 0 and 1.5 ± 0.7 CFU-GM/5× 10^4 marrow cells, respectively, in methylcellulose cultures

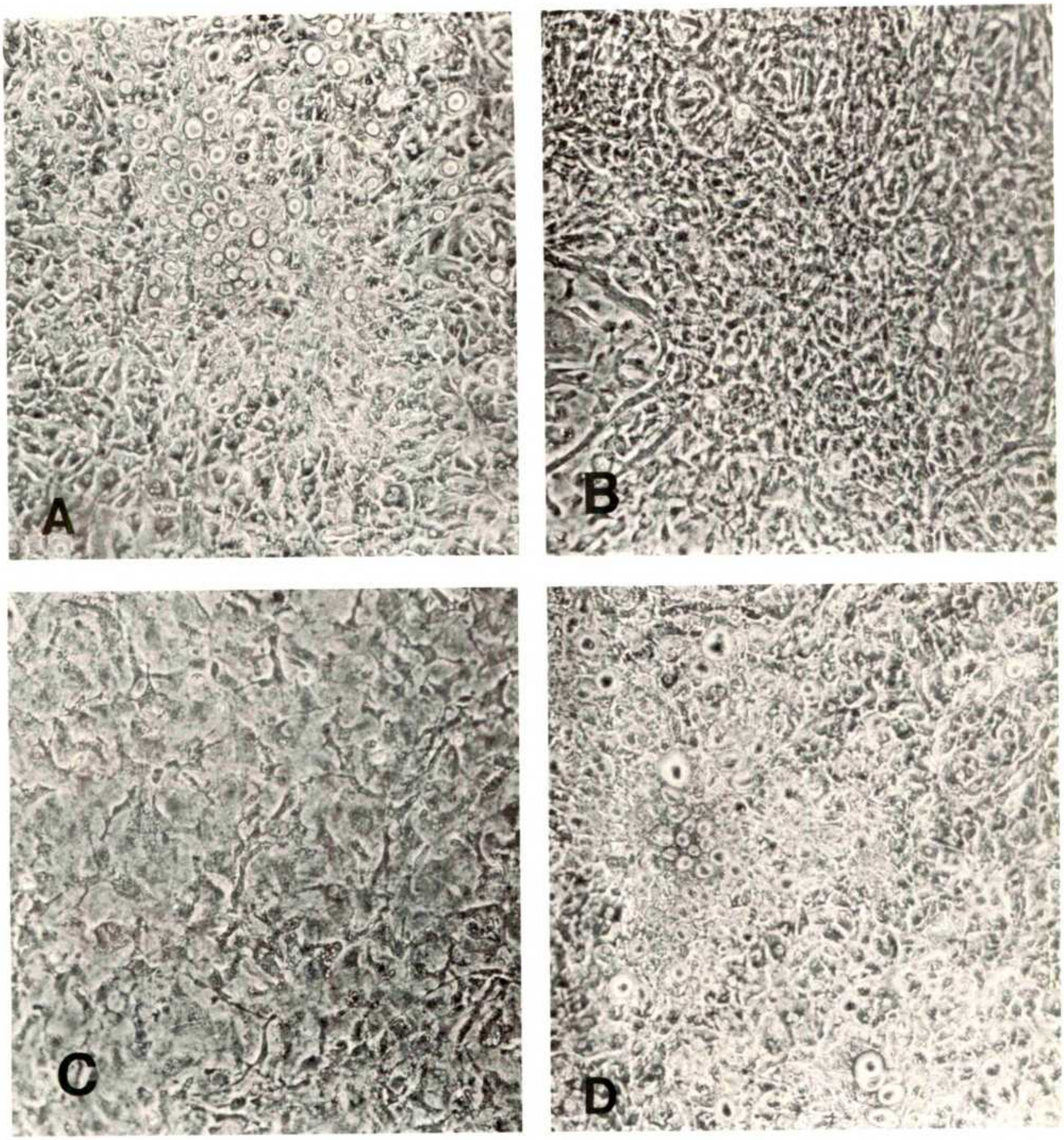

Fig 1. Photomicrographs of stromal cells in myeloid and lymphoid long-term bone marrow cultures after MPA treatment. (A) Stroma from myeloid bone marrow culture initiated and maintained under MBMC conditions. (B) Stroma from myeloid bone marrow culture after 2 weeks under LBMC culture conditions. (C) Stroma from lymphoid bone marrow culture initiated and maintained under LBMC culture conditions. (D) Stroma from lymphoid bone marrow cultures after 2 weeks under MBMC culture conditions. All micrographs ×500.

hematopoietic cells present in primary LBMC that might potentially occupy niches required by precursors in the reseed population. An additional advantage of adding a known number of cells and progenitors to purified stroma was that cell growth could be quantitated at weekly intervals thereafter relative to that starting population. Separate aliquots of 5×10^5 nylon wool-passed cells were cultured alone in quadruplicate to ensure that no stromal cells present in that population grew in vitro.

Table 2 shows the results pooled from two experiments in which nylon wool bone marrow was seeded onto MPA-treated stroma initiated under LBMC conditions. The data indicate that this stroma can support myelopoiesis under MBMC conditions. By 2 weeks after seeding, a 27-fold increase in the number of nonadherent cells per flask was observed, and at least a 16-fold increase was maintained through the 7 weeks of observation. Progenitors of granulocytes and macrophages (CFU-GM) were also present. While

Table 2. Stroma Grown Under LBMC Culture Conditions Can Support Myelopoiesis on Transfer to MBMC Conditions

Wk of Culture	Cells per Flask × 10^6	CFU-B/ 5 × 10^4	CFU-GM/ 5 × 10^4	Mean No. CFU-GM per Flask
0	0.5	29 ± 17	213 ± 15	2,130
1	2.1 ± 0.6	0.3 ± 0.3	125 ± 71	5,000
2	13.9 ± 1.6	0	20 ± 6	5,560
3	11.7 ± 3.0	0	19 ± 6	4,446
4	11.8 ± 3.0	0	21 ± 13	4,956
5	11.2 ± 3.6	0	15 ± 10	3,584
6	8.5 ± 3.0	0	16 ± 12	2,720
7	8.2 ± 2.0	0	14 ± 12	2,296

Stroma from SCID bone marrow was grown under LBMC culture conditions, treated with MPA, seeded with 5 × 10^5 2× nylon wool-passed BALB/c bone marrow cells, and transferred to MBMC culture conditions. Half of nonadherent cells were removed each week and the assays noted were performed. Data pooled from two separate experiments in which a total of seven cultures were processed individually. Values are expressed as mean ± SD.

the frequency of these was below the level in the initial reseed population, their absolute number was always greater through the course of the experiment. No hemopoiesis was observed in replicate MPA-treated LBMC stromal cell cultures not reseeded but transferred to MBMC conditions or in MPA-treated LBMC maintained under LBMC conditions, confirming the efficacy of the MPA treatment. Four separate cultures, each initiated with 5 × 10^5 nylon wool-passed cells alone, failed to establish stromal cell foci or confluent adherent layers; hemopoiesis did not initiate in these flasks, and cells did not survive past 1 week (data not shown).

B lymphocytes are not produced under MBMC conditions, and consistent with this was the fact that no CFU-B were observed in the cultures by 2 weeks postseeding (Table 2). However, a primitive B cell precursor is present in primary MBMC.[16] Whether or not they were present in the reseeded LBMC transferred to MBMC conditions was tested by harvesting cells from the adherent layer of the reseeded cultures and transplanting them into SCID mice. The data in Table 3 demonstrate that B cells are present in SCID mice that received a graft of the cultured cells. Higher levels of CFU-B and surface IgM-positive cells were present in recipients of cultures seeded 4 weeks previously as compared with 7 weeks earlier; however, because no B cells are present in unreconstituted SCID mice, reconstitution to any level is significant. No evidence of tumor formation was noted in any of the mice. Because they had received adherent layer cells containing both stromal and hemopoietic components, this suggests that both populations are normal. Taken together, these results demonstrate that LBMC stroma can support myelopoiesis and maintain B cell precursors.

MBMC stroma can support lymphopoiesis after transfer to LBMC conditions. The capacity of stroma initiated under MBMC conditions to support lymphopoiesis was tested by treating MBMC with MPA, seeding them with 5 × 10^5 nylon wool-passed BALB/c bone marrow cells, and transferring cultures under LBMC conditions to a 37°C incubator. As in the previous section, the number of progenitors in the nylon wool population was determined, thus making it possible to quantify cell growth at weekly intervals relative to the reseed population.

The data in Table 4 were pooled from four separate experiments and demonstrate that MBMC stroma can support B lymphopoiesis. At all time points examined, surface IgM-positive cells and CFU-B were present in the cultures. Those cells that did not express Ig were morphologically identifiable as lymphocytes. Myeloid progenitors are not present in LBMC by the first few weeks postinitiation, and the results of these experiments show that the number of CFU-GM decrease to 10% of input levels by 2 weeks postseeding and are not detectable at 6 weeks.

Negative controls similar to those described earlier were

Table 3. Stroma Grown Under LBMC Culture Conditions Can Support Early B Cell Precursors on Transfer to MBMC Conditions

Cells Transplanted Into SCID Mice	Spleen Cell No. × 10^6	sIgM$^+$ (%)*	CFU-B/10^5	Absolute No. CFU-B
None	6.3†	0	0	0
Experiment 1				
3 × 10^6 4 wk post-reseed	32	16.0	205 ± 7	6.6 × 10^4
	37	29.8	280 ± 28	1.0 × 10^5
Experiment 2				
10^7 4 wk post-reseed	33	ND	115 ± 35	3.8 × 10^4
	22	ND	385 ± 21	8.5 × 10^4
Experiment 3				
10^7 7 wk post-reseed	12	1.2	4 ± 2	4.8 × 10^2
	12	1.2	22 ± 8	2.6 × 10^3
Experiment 4				
10^7 7 wk post-reseed	27	1.4	2 ± 1	5.4 × 10^2
	18	0	5 × 0.5	9.0 × 10^2

Stroma from SCID bone marrow was grown under LBMC conditions, treated with MPA, seeded with 5 × 10^5 2 × nylon wool BALB/c bone marrow cells, and transferred to MBMC conditions; cells were harvested for transplantation into SCID mice at times noted. Values are expressed as mean ± SD.

Abbreviation: ND, not determined.

*Based on counts of 500 cells.

†Data were pooled from six unreconstituted SCID mice.

Table 4. Stroma Grown Under MBMC Culture Conditions Can Support B Lymphopoiesis on Transfer to LBMC Conditions

Wk of Culture	Cells per Flask $\times 10^6$	CFU-GM/ 5×10^4	sIgM$^+$ (%)	CFU-B/ 5×10^4	Mean No. CFU-B/Flask
0	0.5	165 ± 57	0.4 ± 0.3	26 ± 10	2.6×10^2
1	3.8 ± 3.4	48 ± 24	7.5 ± 2.0	120 ± 85	9.0×10^3
2	1.6 ± 1.2	15 ± 11	14.0 ± 9.0	500 ± 402	1.6×10^4
3	0.9 ± 0.4	10 ± 13	13.6 ± 6.0	117 ± 75	2.1×10^3
4	1.0 ± 0.3	10 ± 7	14.0 ± 4.0	74 ± 78	1.5×10^3
5	1.3 ± 0.4	13 ± 14	10.0 ± 8.0	28 ± 35	7.3×10^2
6	1.7 ± 0.5	0	9.8	90 ± 20	3.1×10^3

Stroma from SCID bone marrow was grown under MBMC culture conditions, treated with MPA, seeded with 5×10^5 2 × nylon wool-passed BALB/c bone marrow cells, and transferred to LBMC conditions. Half of nonadherent cells were removed each week and the assays noted were performed. Data was pooled from four separate experiments in which a total of 13 flasks were processed individually. Values are expressed as mean ± SD.

included in all experiments. No hemopoiesis initiated in unseeded, MPA-treated MBMC transferred to LBMC conditions or maintained under MBMC conditions. Four replicate cultures, each containing 5×10^5 nylon wool cells seeded into empty flasks under LBMC conditions, did not form an adherent layer and cells did not survive past 1 week

DISCUSSION

The availability of different LTBMC that support myelopoiesis or lymphopoiesis in a stromal cell-dependent system offers an in vitro means of investigating the formation of the hemopoietic microenvironment. Using populations of stromal cells depleted from hemopoietic cells grown under MBMC or LBMC conditions, obtained by treating established cultures with MPA,[24] it has been possible to compare the stromal cell populations present in the two cultures. Results indicate that the different culture conditions affect stromal cell physiology in a manner that may contribute to the pattern of hemopoiesis in the respective cultures.

The analysis of LBMC demonstrated that the stroma that establishes in those cultures has the capacity to support long-term myelopoiesis after seeding with nylon wool-passed bone marrow cells and transfer to MBMC conditions. The lack of myelopoiesis in primary LBMC does not reflect a differentiation block of myeloid stem cells that may be present in the cultures; CFU-S are not present,[20] and the transfer of established LBMC to MBMC conditions results in a cessation of lymphopoiesis and no initiation of myelopoiesis.[29] The lack of myelopoiesis in LBMC is also not due to toxic conditions, since the culture medium contains the same basic components used to grow myeloid progenitors in vitro.[27] The simplest explanation would be that culture conditions are suboptimal for the survival and differentiation of myeloid cells. The present findings suggest that this is due in part to the failure of the stroma to form the proper microenvironment and produce myeloid growth factors. The ability of LBMC stroma to support myelopoiesis under MBMC conditions may be due in part to the initiation of myeloid growth factor production by the stroma.

The capacity of MBMC stroma to support B lymphopoiesis was consistent with results that demonstrated that B lymphopoiesis could be induced and maintained in MBMC by transferring an established culture to LBMC conditions.[30] While the significance of that study was the induction of B lymphopoiesis from early hemopoietic cells in vitro, it also demonstrated that MBMC stroma was not lineage restricted in its support capacity. The high concentration of steroids and horse serum used in MBMC might be inhibitory to B lymphopoiesis.[17] A contributing factor to the lack of lymphopoiesis in the cultures may also be that the stromal cells only secrete myeloid growth factors and not those on which lymphopoiesis may be dependent.[31,32]

The present results support other reports that stromal cells are sensitive to external stimuli. Lanotte et al[28] demonstrated that steroids present in the culture medium resulted in an accumulation of lipids in a stromal cell line. Because steroids are present in MBMC medium, the appearance of lipid in LBMC stromal cells transferred to MBMC conditions may also be a steroid-mediated effect. Cohen et al[33] also noted effects of steroids on stromal cells; lipid-containing cells were present in steroid-containing cultures, while those established in fetal calf serum without steroids had primarily a fibroblastoid appearance. That study also demonstrated that the stromal cell cultures established without steroids could support myeloid progenitors after seeding with purified populations of hemopoietic cells. The exogenous steroids in LTBMC do not appear to affect growth factor secretion. MBMC stromal cells grown in horse serum-supplemented medium without hydrocortisone secreted myeloid growth factors that stimulated comparable numbers of CFU-GM as in steroid-supplemented cultures.[29] Other components of the culture medium, such as glucose,[34] may also be of importance in affecting stromal cell function.

It is unclear whether the stromal cells initiated under LBMC or MBMC conditions are identical or different populations. It is possible that at least two distinct stromal cell subpopulations, one for myelopoiesis and one for lymphopoiesis, are present in each of the LTBMC. That population necessary for myelopoiesis would be functional under MBMC but not LBMC conditions. Those central to lymphophoiesis would be functional under LBMC but not MBMC conditions. Even if such distinct subpopulations exist in the cultures, their activation into functional cells might still be affected by environmental conditions. Alternatively, the stromal cells in the two cultures may be identical and exhibit a plasticity in response to culture conditions. Under MBMC conditions they form myeloid microenvironments, while under LBMC conditions they only support lymphopoiesis.

It has been suggested that there are gradients of metabolites and cell products that exist within the medullary cavity, thereby producing intraorgan variability in environmental conditions.[35] If this is the case, then the capacity of stromal cells to modulate their function according to differences in their external milieu could have relevance to the type of microenvironment they form in a particular area. For example, it has been suggested that stem cells are localized to subendosteal regions.[3] In this case the localized conditions in those areas might be optimal not only for stem cells themselves, but also to the function of stromal cell populations that form stem cell niches.

REFERENCES

1 Osmond DG, Batten SJ: Genesis of B lymphocytes in the bone marrow. Extravascular and intravascular localization of surface IgM bearing cells in mouse bone marrow detected by electron-microscope radioautography after *in vivo* perfusion of ^{125}I anti-IgM antibody Am J Anat 170:349, 1984

2. Lambertsen RH, Weiss L: A model of intramedullary hematopoietic microenvironments based on stereologic study of the distribution of endoclonal marrow colonies Blood 63:287, 1984

3. Lord BI: Cellular and architectural factors influencing the proliferation of hematopoietic stem cells, in Clarkson B, Marks P, Till J (eds) Differentiation of Normal and Neoplastic Hematopoietic Cells Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1978, p 775

4. Wolf NS, Trentin JJ. Hemopoietic colony studies V Effect of hemopoietic organ stroma on differentiation of pluripotent stem cells J Exp Med 127:205, 1968

5. Wolf NS: The hemopoietic microenvironment. Clin Haematol 8:469, 1979

6 Weiss L: The structure of bone marrow J Morphol 117.467, 1965

7. Lichtman MA: The ultrastructure of the hemopoietic environment of the marrow: A review. Exp Hematol 9 391, 1981

8. Tavassoli M Ultrastructural and radiobiological characterization of stromal cells in continuous long-term marrow culture, in Johari O (ed): Scanning Electron Microscopy 1982/1. Chicago, SENS Inc. AMF O'Hare, 1982, p 349

9. Zipori D, Duskin D, Tamir M, et al: Cultured mouse marrow stromal cell lines II Distinct subtypes differing in morphology, collagen types, myelopoietic factors, and leukemic cell growth modulating activities. J Cell Physiol 122:81, 1985

10. Dorshkind K, Witte ON Long-term murine hemopoietic cultures as model systems for the analysis of B cell differentiation. Contemp Top Microbiol Immunol (in press)

11. Dexter TM, Allen TD, Lajtha LG: Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. J Cell Physiol 91:335, 1977

12. Jones-Villeneuve E, Phillips RA: Potentials for lymphoid differentiation by cells from long-term cultures of bone marrow. Exp Hematol 8 65, 1980

13 Schrader JW, Schrader S. *In vitro* studies on lymphocyte differentiation. I. Long-term *in vitro* culture of cells giving rise to functional lymphocytes in irradiated mice. J Exp Med 148.823, 1978

14 Aspinall R, Owen JJT: An investigation into the B lymphopoietic capacity of long-term bone marrow cultures. Immunology 48:9, 1983

15. Nishikawa S, Kina T, Amagi T, et al: Early B cell precursors in long-term bone marrow culture. Selective development in the bone marrow of irradiated recipients Eur J Immunol 15.696, 1985

16. Dorshkind K, Phillips RA: Characterization of early B lymphocyte precursors in long-term bone marrow cultures. J Immunol 131:2240, 1984

17 Whitlock CA, Witte ON: Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci USA 79.3608, 1982

18 Dorshkind K, Schouest L, Fletcher WH: Morphologic analysis of long-term bone marrow cultures that support B-lymphopoiesis or myelopoiesis. Cell Tissue Res 239:375, 1985

19. Phillips RA, Bosma MJ, Dorshkind K: Reconstitution of immune-deficient mice with cells from long-term bone marrow cultures, in Wright, DG, Greenberger JS (eds): Long-Term Bone Marrow Culture. New York, Liss, 1985, p 309

20. Kurland JI, Ziegler SF, Witte ON. Long-term cultured B lymphocytes and their precursors reconstitute the B-lymphocyte lineage *in vivo* Proc Natl Acad Sci USA 81:7554, 1984

21 Phillips RA: Enhanced lymphoid and decreased myeloid reconstituting ability of stem cells from long-term cultures of mouse bone marrow J Supramol Struct 14:77, 1980

22. Bosma GC, Custer RP, Bosma MJ: A severe combined immunodeficiency mutation in the mouse. Nature 301:527, 1983

23. Dorshkind K, Keller GM, Phillips RA, et al: Functional status of cells from lymphomyeloid tissues in mice with severe combined immunodeficiency disease J Immunol 132:1804, 1984

24 Dorshkind K, Johnson A, Collins L, et al: Generation of purified stromal cell cultures that support lymphoid and myeloid precursors. J Immunol Methods 89:37, 1986

25. Julius MH, Simpson E, Herzenberg LA: A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur J Immunol 3:645, 1973

26. Kincade PW, Paige CJ, Michael R, et al: Characterization of murine colony-forming B cells. I. Distribution, resistance to anti-immunoglobulin antibodies, and expression of Ia antigens J Immunol 120.1289, 1978

27 Iscove NN: Culture of lymphocytes and hemopoietic cells in serum free medium, in Barnes DW, Sirbasku DA, Sato GH (eds) Methods for Serum-Free Cultures of Neuronal and Lymphoid Cells. New York, Liss, 1984, p 169

28 Lanotte M, Scott D, Dexter TM, et al: Clonal preadipocyte cell lines with different phenotypes derived from murine marrow stroma: Factors influencing growth and adipogenesis *in vitro*. J Cell Physiol 111.177, 1982

29 Dorshkind K: Unpublished observations, November, 1985

30. Dorshkind K: In vitro differentiation of B lymphocytes from primitive hemopoietic precursors present in long-term bone marrow cultures. J Immunol 136:422, 1986

31. Landreth KS, Engelhard D, Bear MH, et al: Regulation of human B lymphopoiesis Effect of urinary activity associated with cyclic neutropenia J Immunol 134.2305, 1984

32. Palacios S, Henson G, Steinmetz M, et al. Interleukin 3 supports growth of mouse pre-B cell clones *in vitro*. Nature 309:126, 1984

33. Cohen GI, Cannelos GP, Greenberger JS: In vitro quantitation of engraftment between purified populations of bone marrow hemopoietic stem cells and stromal cells, in Gale RP (ed): Biology of Bone Marrow Transplantation, Proc ICN-UCLA Symp Autologous Bone Marrow Transplantation. Orlando, FL, Academic, 1980, p 491

34 Zipori D, Sasson T Myelopoiesis in the presence of stromal cells from mouse bone marrow. II. Mechanism of glucose dependent colony formation. Exp Hematol 9:663, 1981

35. Metcalf D, Moore MAS: Haemopoietic Cells. Frontiers of Biology Elsevier Amsterdam, North Holland, 1971, p 313

Classifying Acute Leukemia by Immunophenotyping: A Combined FAB-Immunologic Classification of AML

By Peter B. Neame, Praniti Soamboonsrup, George P. Browman, Ralph M. Meyer, Ann Benger, W. Edwin C. Wilson, Irwin R. Walker, Niloufer Saeed, and John A. McBride

A panel of commercially available monoclonal antibodies and five heteroantisera were used to distinguish and subtype 138 cases of acute leukemia (AL). The immunophenotype was compared with the French-American-British (FAB) classification obtained on the cases. The immunophenotype discriminated acute myelogenous leukemia (AML) from acute lymphoblastic leukemia (ALL) and recognized cases not distinguished by cytochemistry (22% of cases), mixed lineage phenotypes (13% of cases), and cases with separate populations of lymphoblasts and myeloblasts (one case). Using the immunologic panel and derived criteria to subtype AML, correspondence of the immunophenotype to the FAB subtypes M1, M2, M4, and M5 was possible in greater than 80% of cases. A combined classification of the immunophenotype and FAB morphology/cytochemistry was devised for AML subtyping. It is recommended that immunophenotyping should be done at least in all cases with negative or inconclusive cytochemistry. At present, we suggest that until a "gold standard" for identifying leukemic subtypes is developed, the best method for typing acute leukemia is by using a combination of morphology, cytochemistry and immunophenotyping.

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THE FRENCH-AMERICAN-BRITISH (FAB) classification of acute leukemia was proposed in 1976 to standardize the morphologic and cytochemical classification of acute leukemia so that comparisons could be made between institutions and between various trials.[1] Romanowsky morphology and cytochemical stains were used, which included myeloperoxidase (MPO) and Sudan Black B (SBB) for granulocytic differentiation and nonspecific esterase (NSE) for monocytic differentiation.[2] Initially nine subtypes were identified: six myeloid (M1 through M6) and three lymphoid (L1 through L3) but a further megakaryoblastic subtype M7 (as distinct from M7 [M1 + 2 + 4])[3,4] was added in 1985, though methods in addition to light microscopy were used for identification.[5,6] In the interim various heteroantisera and monoclonal antibodies had been used to classify acute leukemia.[7–14] Difficulties with FAB diagnostic criteria had also become apparent.[3,4] The FAB investigators endeavored to improve their classification by introducing revisions in 1981[15] and in 1985[16]; however, some difficulties remain in interpretation.[17] Instituting an immunologic classification for comparative purposes had potential problems as different laboratories used various monoclonal antibodies and heteroantisera.[7–14,18–24] Also, a myeloid subclassification corresponding to the FAB classification has not been well-defined using immunological reagents.

In this paper we describe how a panel of commercially available monoclonal antibodies and five heteroantisera were evaluated for distinguishing and subtyping acute leukemic cells. The purpose of our study was first to select from readily available immunologic reagents a useful panel of monoclonal antibodies and heteroantisera to classify acute leukemia so that comparisons could be made between various laboratories using similar reagents. Secondly, we wished to compare the immunophenotype obtained on each case with its corresponding FAB diagnosis. In particular we were interested in determining whether an immunophenotypic pattern corresponding with the FAB myeloid subtypes could be selected from the panel of monoclonal antibodies used. Our third aim was to derive, if possible, a combined FAB-immunological classification to subtype acute leukemia. In another paper we present a formal evaluation of the contribution of the immunophenotypic classification to the diagnostic concordance of leukemic subtypes.[25]

MATERIAL AND METHODS

Morphologic, Cytochemical and Immunological Studies

The peripheral blood and bone marrows of 138 patients (122 adults, 16 children) with acute leukemia (AL) diagnosed over 2½ years, between December 1983 and June 1986, were tested for surface membrane, cytoplasmic, and nuclear antigens and were classified by the FAB Cooperative Group classification using Wright-stained smears and cytochemical stains Cases of chronic myelogenous leukemia (CML) in blast crisis were not included in this study The cytochemical stains used included periodic acid Schiff (PAS), SBB, MPO, nonspecific esterase-butyrate (NSE), and in some cases chloracetate esterase (CAE) and acid phosphatase (AP) [26] According to the number of cells available, testing was done with the monoclonal antibodies and heteroantisera shown in Table 1 by using immunofluorescence microscopy. The monoclonal antibodies were selected for testing on the basis of previously reported type specificity.[7,9–11,18,27–41] Some antibodies (AML2.23, PMN6, PMN29, FMC10, Leu M3, P1M1, and PLT1) were added as they became available.

FAB Classification

The FAB Cooperative Group classification[1,2,15] with minor modification was used on the peripheral blood and bone marrow smears. The myeloid leukemias were subclassified according to the following criteria:

M1. Predominance of myeloblasts with <10% granulocytic differentiation

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Table 1. Antibodies

Antibody	Cluster Designation (CD Number)[59,71-73]	Molecular Weight (kilodaltons, kd)[59,71-73]	Specificity	Source	Ref.
Leu 1	5	67	T lineage	Becton Dickinson	27
T3, T4, T6, T8, T11	3, 4, 1, 8, 2	19-29, 55, 45/12, 32, 50	T lineage	Coulter Immunology	28
OKT9	NA	90	T lineage	Orthodiagnostic	28
B1, B2, B4	20, 21, 19	35, 140, 95	B lineage	Coulter Immunology	12
BA1, BA2	24, 9	45/55/65, 24	B lineage	Hybritech	29
J5	10	100	Calla	Coulter Immunology	7, 12
HLA-DR (Ia)	NA	29-34	B lineage, monocytic, myeloblast	Becton Dickinson	30
Leu M1	NA	NA (x-hapten)	Granulocytic, monocytic	Becton Dickinson	31
My7, My9, Mol	w13, NA, 11	160, 68-74, 155/94	Granulocytic, monocytic	Coulter Immunology	10, 32, 37
My4, Mo2	w14, w14	55, 55	Monocytic	Coulter Immunology	10, 33
AML 2 23	NA	NA	Granulocytic, monocytic	Hybritech	9
Leu M3	NA	NA	Monocytic	Becton Dickinson	11
D5D6(CAML1)	NA	NA	Granulocytic, monocytic	UCLA Tissue Typing Lab	18
PMN 6, PMN 29	NA, NA	NA, NA	Granulocytic	Hybritech	9
FMC10	15	NA	Granulocytic	AMD-Cedarlane	34
PLM1	NA	NA	pl. GP IIb/IIIa megakaryocytic	AMD-Cedarlane	35, 36
PLT1	NA	NA	Megakaryocytic	Coulter Immunology	37
FVIIIR	NA	NA	Megakaryocytic	Cappel	36, 38
EMcAb*	NA	NA	Erythroid/Lymphoid	B.S. Clarke McMaster University	
TdT†	NA	NA	Lymphoid	Pharmacia	39
Antihuman Immunoglobulin†, μ chain, kappa and lambda light chain	NA	NA	B lineage	Tago	40, 41

*Erythroid monoclonal antibody—not commercially available

†Polyclonal.

NA, Not available.

M2 More than 30% myeloblasts with >10% differentiating granulocytes, NSE <20%.

M3: (a) Hypergranular promyelocytes with numerous Auer rods on Wright-stain or CAE (b) A variant form showing cells with bilobed, multilobed or reniform nuclei (NSE-negative) and relative scarcity of hypergranular promyelocytes or of primitive cells with multiple Auer rods.

M4: Monocytic cells with >20% but <80% NSE-butyrate positivity.

M5. Monocytic cells with >80% NSE positivity. (*a*) Monoblastic, poorly differentiated. (*b*) Monocytic, differentiated

M6: More than 50% erythroblasts with >30% myeloblasts excluding the erythroid cells.

The criteria used for the myeloid subclassification were similar to the revised FAB classification recently published[16] and were introduced in our laboratory in 1983 as a result of the FAB reports in 1981[15] and 1982[42] and from examination of our data.

Cell Preparation and Immunofluorescence

Monoclonal antibodies This has been described in detail in a previous paper.[26] In brief: after Ficoll-Hypaque gradient separation, cells were incubated for 30 minutes with the monoclonal antibody (McAb). After three washes the cells were then incubated for 30 minutes with fluoroscein-conjugated antimouse immunoglobulin To increase the reactivity of some monoclonal antibodies (My9, My7, My4, Leu M1, PMN 6/29, and B4) biotin-conjugated antimouse immunoglobulin was used as the second antibody and for the third stage fluorescein conjugated avidin was added After three further washes, cytospin preparations were made and examined under the fluorescent microscope.

Heteroantisera

Surface immunoglobulin. Cells were incubated with fluorescein-conjugated antihuman immunoglobulin for 30 minutes After three washes, cytospin preparations were made and examined using a fluorescent microscope.[40]

Cytoplasmic immunoglobulin After fixing for five minutes in acetone, cytospin preparations of cells were incubated for 30 minutes with fluorescein-conjugated antihuman immunoglobulin. After three washes, the preparations were examined using a fluorescent microscope.[41]

Terminal deoxynucleotidyl transferase An indirect immunofluorescent assay using rabbit anti–terminal deoxynucleotidyl transferase (anti-TdT) was performed as previously described.[26,39]

Criterion for all methods Cases where more than 25% of mononuclear cells were reactive with antibody were considered positive

Comparison of the Derived Immunophenotype with the FAB Classification

Two experienced observers independently classified (using the FAB classification as described above), randomly ordered, and

Table 2. Reactivity Pattern of Acute Myeloid Leukemia with Monoclonal Antibodies

	Reactivity pattern. No positive/ No analyzed (percent positive)						
FAB (No.)	My9	My7	Leu M1	My4	PMN 6/29	AML 2 23	Ia
M1(18)	14/17 (82)	11/17 (65)	0/18 (0)	1/18 (5)	0/10 (0)	1/6 (17)	9/15 (60)
M2(17)	12/15 (80)	13/15 (87)	15/17 (88)	1/16 (6)	2/10 (20)	1/5 (20)	7/11 (64)
M3(8)	6/6 (100)	6/6 (100)	3/6 (50)	0/7 (0)	4/6 (67)	4/4 (100)	1*/8 (13)
M4(15)	15/15 (100)	13/15 (87)	13/15 (87)	12/15 (80)	7/13 (54)	6/7 (86)	14/15 (93)
M5(14)	11/13 (85)	6/13 (46)	13/14 (93)	14/14 (100)	1/9 (11)	3/4 (75)	13/13 (100)
M6(2)†	1/2	1/1	1/1	—	—	—	—
M7(1)‡	1/1	1/1	0/1	0/1	0/1	0/1	0/1
Total Non-mono (M1, 2, 3, 6)	34/41 (83)	32/40 (80)	19/43 (44)	2/42 (5)	6/27 (22)	6/16 (38)	17/35 (49)
Total mono (M4, 5)	26/28 (93)	19/28 (68)	26/29 (90)	26/29 (90)	8/22 (36)	9/11 (82)	27/28 (96)
Total AML (75)	60/69 (87)	51/68 (75)	45/72 (63)	28/71 (39)	14/49 (29)	15/27 (56)	44/63 (70)
ALL	6/50 (12)	7/50 (14)	5/47 (11)	1/49 (2)	0/19 (0)	2/8 (25)	34/44 (77)

*Promyelocytic variant

†EMcAb > 25%

‡PLT1 and PLM1 > 25%

coded Wright-stained smears together with their cytochemical preparations The results of the formal evaluation of the initial 105 cases are presented in another paper.[25] The concordant FAB results were used for comparison with the results of the immunophenotype. Certain patterns for FAB M1, M2, M4, and M5 using monoclonal antibodies against myeloid differentiation antigens were recognized from our initial data. These patterns were formally tested by reading the immunophenotype of coded cases without knowledge of the FAB diagnosis and then comparing the results with the FAB diagnosis that had been previously recorded by the two observers as described above.

RESULTS

Comparison of the FAB Classification by the Two Observers

Of the 138 cases, there was agreement in the FAB diagnosis of 83% of the cases (113 cases). In the remaining 17% (23 cases), 15 cases showed FAB subtype differences (M1/M2 [4], M1/M5 [1], M2/M4 [2], M4/M5 [2] or L1/L2 [6]) while eight cases were unclassifiable (AUL). In 30 cases (22%), there was negative cytochemical staining. The two observers were concordant in their FAB diagnosis in 20 of these cases. Of the remaining 10 cases, eight were undifferentiated and two were minor subtype differences.

Initial Selection of Antibodies

The reagents used to type acute leukemia are shown in Table 1. Some reagents showed a lack of specificity, poor reactivity, an irregular response to differentiation, or maturation or infrequent positivity in AML and were dropped from our panel. The reagents ultimately used to immunophenotype are shown in Tables 2, 3 and 4.

Relationship Between Immunophenotype and FAB Classification in AML

The reactivity pattern of 75 cases of AML with monoclonal antibodies against myeloid differentiation antigens is shown in Table 2. It will be noted that 87% of the cases were identified by My9 and 75% by My7.[10] Of these 75 cases, 12 were My7$^-$ and My9$^+$; five My7$^+$ and My9$^-$; and three My7$^-$ and My9$^-$. The remaining 55 cases (73%) were positive for both antibodies (My7$^+$, My9$^+$). Ninety-six percent of the cases of AML expressed either My9, My7, or both.[32] Three of the 75 cases that failed to be identified by My7 and My9 were later recognized by other criteria to be myeloid in origin. In two of the patients, the bone marrows were considered to be M5 by morphology and NSE reaction (>80%) and were both My4-positive. A further case, SBB- and MPO-positive, was negative with all myeloid antibodies used (My7$^-$, My9$^-$, My4$^-$, and Leu M1$^-$).

From surveying our initial results we recognized that both My7 and My9 antibodies would be useful as an initial screen for acute myeloid leukemia. Pooling My7 and My9 antibodies was first undertaken when insufficient cells had been obtained by marrow aspirate or on peripheral blood. In 20 further cases pooled My7/My9 antibodies were compared with the results when the reagents were used individually. Using the pooled reagents, an increase in the intensity of the reaction, and sometimes in the number of positive cells, was observed in the majority of cases. In a similar manner, we now pool Leu 1 and T11 (see Table 4).

Table 2 shows that the immunophenotyping can distinguish between FAB M1 and M2. None of the 18 cases classified as FAB M1 by morphological examination were Leu M1–positive, whereas FAB M2 were positive in 15 of 17 cases (88%).

The reactivity of the reagent AML 2.23 has not previously been reported in acute promyelocytic leukemia (FAB M3).[9]

Table 3. Reactivity Pattern of ALL with Monoclonal Antibodies

Type (No)	B4	J5	T11	Leu 1	TdT*	Ia
Null† (6)	6/6	0/6	0/6	0/6	6/6	6/6
Common (26)	26/26	26/26	0/24	0/23	25/25	20/21
T (13)	0/13	3/13	13/13	12/13	13/13	3/13
B‡ (6)	6/6	3/5	0/6	0/6	0/6	4/4
Total Non-T (38)	38/38	29/37	0/36	0/35	31/37	30/31
Total T (13)	0/13	3/13	13/13	12/13	13/13	3/13
AML	0/79	0/75	3/76	0/76	2/86	43/63

*Heteroantiserum.

†cALLa negative Non-T, Non B-ALL.

‡SIg$^+$

Table 4. Monoclonal Antibodies for Classifying Acute Leukemia

	Acute Myeloid Leukemia	Acute Lymphoid Leukemia		
First Line	My 7/9	TdT*	B4	Leu1/T11†
2nd Line‡	Leu M1, My4, EMcAb§, Ia, PMN 6/29, AML 2.23	Ia	J5, cμ* SIg*	T3, T6, T9
3rd Line‡	FVIIIR, PLT1 Gp IIb/IIIa		Kappa* Lambda* B1, B2	T4, T8

*Heteroantiserum.

†An addition would be Leu 9, (CD7, 40 kd) [73]

‡Second and third line subsequently performed according to lineage established by the first line

§EMcAb, erythroid monoclonal antibody; not commercially available.

We found that all four cases of FAB M3 including a variant form[43] gave a positive reaction with AML 2.23. All of these cases were My4$^-$ but the variant form was Ia$^+$ in contrast to the hypergranular form which was Ia$^-$. The combination of Ia$^-$, AML 2.23$^+$, and My4$^-$ was not observed in FAB M1, M2, M4, or M5.

As previously reported,[10] My4 reacted positively in the majority (90%) of cases of FAB M4 and M5. It gave a positive reaction in only two of 42 cases (5%) classified as nonmonocytic by the FAB classification and a negative NSE reaction. In seven cases NSE-butyrate and My4 reactivity were not concordant.

Eighty-six cases which typed as AML were tested with anti-TdT and two cases were positive. One of the cases was also Tll- and sheep red cell receptor–positive and was considered to be a case of acute mixed lineage leukemia (AMLL).[26,44-47] Two further cases diagnosed as AML by the FAB classification showed myeloid markers plus a single lymphoid marker (Tll) on immunophenotyping[44] (See Table 3).

By observing certain patterns using monoclonal antibodies against myeloid differentiation antigens, a myeloid immunophenotype corresponding to the FAB myeloid subclassification was recognized for M1, M2, M4, and M5 (Table 5).[48] Figure 1 shows that by the immunophenotypic criteria, 85% of cases diagnosed as M1, 93% as M2, 81% as M4 and 100% as M5 corresponded to their appropriate FAB morphologic diagnosis The hypergranular FAB M3 appeared to be Ia$^-$, AML 2.23$^+$, and My4$^-$, but our numbers are too small to draw a firm conclusion Criteria for the diagnosis of M6[49] and M7[35,36] have been published in the literature.

Table 5. Myeloid Immunophenotypic Subclassification

M1	My7/9-positive, Leu M1- and My4-negative
M2	My7/9-positive, Leu M1-positive, My4-negative
M3a*	Ia-negative, AML 2 23-positive, My4-negative
M4	(a) My4-positive but less than 45% (b) Grey zone My4 (between 45-55%) and PMN 6/29-positive
M5	(a) My4-positive greater than 55% (b) Grey zone My4 (between 45-55%) and PMN 6/29-negative

*Too few cases to draw a firm conclusion.

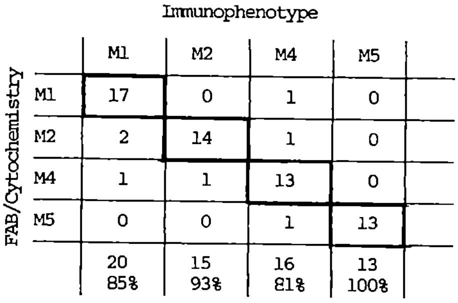
Relationship Between Immunophenotype and FAB Classification in ALL

As previously demonstrated, there was a lack of correlation between the FAB L1, L2 classification and the ALL immunophenotype.[2,13,15] Furthermore, one case classified as FAB L3 showed no SIg positivity and typed as cALL (B4$^+$, J5$^+$, Cμ^-, SIg$^-$).[50,51]

Our immunophenotypic results with acute lymphoid leukemia correspond to those reported in the literature.[8,12,29,52,53] The reactivity pattern with monoclonal antibodies of 51 patients classified as ALL are shown in Table 3. B4 was specific for non–T lineage ALL being positive in all patients tested, and negative in 13 cases of T-ALL. Anti-TdT was positive in all except six cases of B-ALL showing positive SIg. Because BA2 was positive in four out of nine cases of AML and two out of three cases of T-ALL, and because BAl was considered less reliable than B4 for subtyping Null ALL, both antibodies were dropped from our panel.[54] Twelve cases (24%) diagnosed as ALL by the FAB classification showed lymphoid markers and a myeloid marker on immunophenotyping.[46]

Cases With Disagreement Between the Morphological Diagnosis and the Immunophenotype

Table 6 shows the results of 14 cases where there was disagreement between the morphologic diagnosis and the immunophenotype. Thirteen of the cases were undifferentiated by cytochemical staining. Eight of the cases were originally unclassifiable (AUL, M1, or L2) by morphology.



FAB/Cytochemistry	Immunophenotype M1	M2	M4	M5
M1	17	0	1	0
M2	2	14	1	0
M4	1	1	13	0
M5	0	0	1	13
	20 85%	15 93%	16 81%	13 100%

Fig 1. Comparison of results of the immunophenotypic myeloid subclassification and the FAB-cytochemical classification.

Table 6. Laboratory Data of 14 Patients with Disagreement Between FAB Diagnosis and Immunophenotype

No	FAB	Cytochemistry	Positive Immunologic Markers	Immunophenotype	Final Diagnosis
1	M1	Myeloid	Undifferentiated	UD*	AML
2	M1	UD	TdT, B4, J5	Common ALL	ALL
3	AUL†	UD	TdT, B4, J5, B1, BA2	Common ALL	ALL
4	AUL	UD	TdT, B4, BA2, My7	Null ALL (plus My7)	ALL
5	L2	UD	My9, My7, My4	AML-M4	AML
6	M1	UD	B4, B1, B2, SIgA, Lambda	B ALL	ALL
7	M1	UD	TdT(36%), B4(20%), Leu M1, My9, My7, My4 Double labelling showed separate populations of lymphoid and myeloid cells	M × L‡	M × L
8	M2	UD	TdT, B4, J5, My7	Common ALL (plus My7)	ALL
9	AUL	UD	TdT, BA1, BA2	Null ALL	ALL
10	AUL	UD	My7, B4, (TdT Neg, Ia Neg)	AML (plus B4)	AML
11	AUL	UD	TdT, B4, J5, Ia	Common ALL	ALL
12	AUL	UD	TdT, B4, J5, Ia	Common ALL	ALL
13	AUL	UD	TdT, B4, J5, Ia	Common ALL	ALL
14	AUL	UD	My7/My9	AML (M1)	AML

*UD, undifferentiated.

†AUL, acute undifferentiated leukemia (M1/L2)

‡M × L, dual population of myeloblasts and lymphoblasts

In the remaining six cases there had been FAB concordance but the result was in disagreement with the immunophenotype. One of these cases showed negative reactivity with all monoclonal antibodies used despite FAB M1 morphology and strong positivity with MPO and SBB. The immunophenotype included three cases with a mixed lineage phenotype[26] and a single case with a dual population of lymphoblasts and myeloblasts (biclonal, biphenotypic).[26,47,55] The final diagnosis for therapeutic purposes of the cases with a mixed lineage phenotype was considered as either ALL or AML according to the predominant lineage observed on combined morphologic, cytochemical, and immunologic assessment.

DISCUSSION

The FAB classification[1,2,15,16] provides a common language for comparing and treating acute leukemia; however, difficulties with some of the diagnostic criteria are evident.[17] The major difficulties include distinguishing M1 from L2 in cases where cytochemical staining is negative[11,13,56] and M1 from M2[3,4] though their distinction has been improved in the recent revised FAB classification.[16]

From our initial investigation, a panel of commercially available antibodies to discriminate and subtype acute leukemia was devised and is used in the Hamilton region (Table 4). Since its initiation, the panel has been tested prospectively on 58 AL cases. It was developed to obtain the maximum of information using the smallest number of tests. In addition, the panel is performed in sequence so that the most useful information is likely to be derived from specimens which contain an insufficient number of cells for complete analysis. The first line screen is used to distinguish acute myeloid from acute lymphoid leukemia and to separate B and T lineage ALL. It will also identify some cases of AMLL. The second panel discriminates a rare case not recognized by the first panel and further subtypes acute myeloid and lymphoid leukemia. Cases not defined by the first two panels are investigated by a third panel which includes antibodies that recognize megakaryocyte/platelet differentiation or further discriminate subtypes of ALL.

The immunophenotype will discriminate cases of acute lymphoid from myeloid leukemia which remain undifferentiated by cytochemistry (22% of cases) and thereby clarifies the majority of the disagreements (M1 *v* L2) noted between observers using the FAB classification. In addition, cases with a mixed lineage phenotype (13% of cases)[26,46,47,57] and, less commonly, dual populations of lymphoid and myeloid cells (<1% of cases)[26,55,58] can be identified. It seems the immunophenotype should be regarded as a reliable method of classifying acute leukemia. Monoclonal antibodies with the same cluster differentiation (CD) number as defined by the First and Second Leukocyte Antigen Workshop[59,71] could possibly be used to replace some of the monoclonal antibodies utilized in our panel; however, despite their having the same CD number, we found differences in the reactivity of My4 and MO2.[72]

The subclassification of acute lymphoblastic leukemia by immunophenotyping is well-documented.[8,12,13,29,51-53] To date all our cases of non-T–ALL have shown B4 reactivity Null ALL was identified in the panel by reactivity with anti-TdT and B4 only, but Anderson et al[12] have observed presumed cases of non-T ALL that were TdT$^+$ and B4$^-$. As previously reported the ALL immunophenotype did not correspond with the FAB classification[2,50,51]

In most previous reports using different antibodies there has been little correlation between the FAB myeloid subtypes and antigenic phenotype in AML[19,23,60]; however, the expression of certain antigens have correlated with the FAB classification.[10,61] Using our immunologic panel and derived criteria to subtype AML, correspondence of the immunophenotype to the FAB subtypes M1, M2, M4, and M5 was

Table 7. Criteria for FAB Myeloid Subclassification Using Morphology, Cytochemistry and Immunophenotype

FAB M1	+/− 90% Blasts
	SBB, MPO >3% and/or My 7/9 >25%
	Very little maturation (<10%), Leu M1 <25%
M2	>30% Blasts with maturation
	>10% More mature myeloid cells, >25% Leu M1
	<20% NSE and <25% My 4
M3a	Hypergranular Promyelocytes
	Multiple Auer rods (Wright-stain, CAE, or phase contrast microscopy)
	AML 2 23 >25%, Ia <25%, My4 <25%
M4	>30% Blasts
	Monocytic differentiation
	NSE >20%, <80% and/or My 4 >25%, <55%
	My 4 between 45-55%, PMN 6/29 >25%
M5	>30% Blasts
	Monocytic differentiation
	NSE >80% and/or My 4 >55%
	My 4 between 45-55%, PMN 6/29 <25%
M6	>30% Blasts (Nonerythroid population)
	>50% Erythroid precursors (Total marrow cells) E McAb positive
M7	>30% Blasts
	PLT1, or PLM1, or FVIIIR >25% blasts positive

possible in over 80% of cases (Fig 1). Using the immunophenotype together with the FAB morphology and cytochemistry (Table 7) it is possible to obtain almost perfect concordance between observers.[25] It should be emphasized that this scheme is based on the testing of a relatively small sample (75 AML cases). Combining FAB M1, M2, and M4 as so-called M7[3,4] is unnecessary when using the combined FAB/immunophenotypic classification as the various myeloid subtypes can be distinguished.

Should all cases of acute leukemia be immunophenotyped? A major difficulty of the FAB classification is encountered in cases with negative cytochemical staining This was found in 30 of our 138 cases (22%) Though our two observers were concordant in 20 of these 30 cases, in five of the concordant observations there was disagreement between the concordant FAB classification and the immunophenotype. Of the remaining ten cases, eight were undifferentiated (M1 or L2). Immunophenotyping may therefore be useful in the cases with negative cytochemistry. Clarifying the diagnosis in this group of patients will allow therapy to be given according to the type of leukemia diagnosed. A controlled study will be required to ascertain whether this results in an improved therapeutic outcome. Acute mixed lineage leukemia is found in 13% of our cases and could rarely be recognized without immunophenotyping. If therapeutic outcome with standard therapy is found to be inferior to outcome in single lineage leukemia in future studies (in progress), an argument could be made for applying immunophenotyping to all cases of AL.

At this time, the immunophenotype cannot be regarded as the "gold standard" for classifying all cases of acute leukemia. Unfortunately, there is no "gold standard." To date, the most widely accepted classification system has been the FAB classification and for this reason the design of this study was to compare the immunophenotyping of acute leukemia with the FAB classification. The purpose of our paper is to provide data which may improve current systems.[17] The process will hopefully help to establish a classification with biologic meaning or prognostic relevance; however, further controlled studies will have to be done to determine whether therapeutic decisions based upon immunophenotyping in adults improve the outcome as is possible in ALL in childhood.[62,63] Such studies cannot be done until the classification is firmly established.

The possibility that the antigenic phenotyping of AML with monoclonal antibodies may identify groups which are of prognostic importance has been recently reported.[60,61] A correlation with My1 reactivity and successful remission induction has been suggested.[60] In addition, Griffin has noted a correlation of My7 reactivity with poor prognosis in AML, an observation noted previously with My4.[61] The FAB classification has only been minimally successful in identifying groups of clinical relevance.[60]

Following the recent introduction of probes to test for rearrangement of the immunoglobulin or T cell antigen receptor genes,[64–69] it seemed that a test would be available to distinguish acute lymphoid from myeloid leukemia with certainty; however, recently immunoglobulin and T cell antigen receptor rearrangement have been noted in cases with the AML phenotype.[46,70] Thus in cases with a somatic rearrangement of the immunoglobulin or T cell antigen receptor gene, acute lymphoid leukemia may be suspected but is not proven. Cases with a germ-line configuration on DNA analysis would suggest nonlymphoid leukemia.[14] The addition of karyotyping to the morphological and immunologic assessment should also contribute to the detailed cellular phenotype in acute leukemia.[14,17]

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REFERENCES

1. Bennett JM, Catavosky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of acute leukaemias French-American-British (FAB) Co-operative group. Br J Haematol 33:451, 1976

2. Galton DAG, Catovsky D, Sultan C, Bennett JM, Gralnick HR (moderator). Classification of acute leukemia. Ann Intern Med 87 740, 1977

3 Head DR, Savage RA, Cerezo L, Craven CM, Bickers JN, Hartsock R, Hosty TA, Saiki JH, Wilson HE, Morrison FS, Coltman CA, Hutton JJ· Reproducibility of the French-American-British classification of acute leukemia The Southwest Oncology Group experience. Am J Hematol 18:47, 1985

4. Head DR, Cerezo L, Savage RA, Craven CM, Bickers JN, Hartsock R, Hosty TA, Saiki JH, Wilson HE, Morrison FS, Cottman CA, Hutton JJ: Institutional performance in application of the FAB classification of acute leukemia The Southwest Oncology Group experience. Cancer 55.1979, 1985

5. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton

DAG, Gralnick HR, Sultan C: Criteria for the diagnosis of acute leukemia of megakaryocytic lineage (M7). A report of the French-American-British co-operative group. Ann Intern Med 103:460, 1985

6 Bloomfield CD, Brunning RD: FAB M7. Acute Megakaryoblastic leukaemia—Beyond morphology. Ann Intern Med 103:450, 1985

7. Greaves MF, Hariri G, Newman RA, Sutherland DR, Ritter MA, Ritz J. Selective expression of common acute lymphoblastic leukemia (gp 100) antigen on immature lymphoid cells and their malignant counterparts. Blood 61:628, 1983

8. Foon KA, Schroff RW, Gale PR: Surface markers on leukemia and lymphoma cells: Recent advances. Blood 60:1, 1982

9. Ball ED, Fanger MW: The expression of myeloid-specific antigens on myeloid leukemia cells: Correlations with leukemia subclasses and implications for normal myeloid differentiation. Blood 61:456, 1983

10 Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF: Surface marker analysis of acute myeloblastic leukemia. Identification of differentiation-associated phenotypes. Blood 62:557, 1983

11. Herrmann F, Komischke B, Odenwald E, Ludwig WD: Use of monoclonal antibodies as a diagnostic tool in human leukemia. I. Acute myeloid leukemia and acute phase of chronic myeloid leukemia Blut 47:157, 1983

12. Anderson KC, Bates MP, Slaughenhoupt BL, Pinkus GS, Schlossman SF, Nadler LM: Expression of human B cell–associated antigens on leukemias and lymphomas: A model of human B cell differentiation. Blood 63:1424, 1984

13. Avnstrom S, Ralfkier E, Clausen N, Plesner T, Hansen NE, Nissen NI: Immunological markers in acute leukemia. Scand J Haematol 34:16, 1985

14 Chan LC, Pegram SM, Greaves MF. Contribution of immunophenotype to the classification and differential diagnosis of acute leukemia. Lancet 1:475, 1985

15. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: The morphological classification of acute lymphoblastic leukaemia. Concordance among observers and clinical correlations. Br J Haematol 47:553, 1981

16. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan G: Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British co-operative group. Ann Intern Med 103:626, 1985

17. Bloomfield CD, Brunning RD: The revised French-American-British Classification of acute myeloid leukemia Is new better? Ann Intern Med 103:614, 1985

18 Linker-Israeli M, Billing RJ, Foon KA, Terasaki PI: Monoclonal antibodies reactive with acute myelogenous leukemia cells J Immunol 127:2473, 1981

19. Van der Reijden HJ, van Rhenen DJ, Lansdorp PM, van't Veer MB, Langenhuijsen MMAC, Engelfriet CP, von dem Borne EGK: A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. Blood 61:443, 1983

20 Andrews RG, Torok-Storb B, Bernstein ID: Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies Blood 62:124, 1983

21. Linch DC, Allen C, Beverly PCL, Bynoe AG, Scott CS, Hogg N. Monoclonal antibodies differentiating between monocytic and nonmonocytic variants of AML Blood 63:566, 1984

22. Majdic O, Bettelheim P, Stockinger H, Aberer W, Liszka K, Lutz D, Knapp W: M2, a novel myelomonocytic cell surface antigen and its distribution on leukemic cells Int J Cancer 33:617, 1984

23. Pessano S, Palumbo A, Ferrero D, Pagliardi GL, Bottero L, Lai SK, Meo P, Carter C, Hubbell H, Lange B, Rovera G: Subpopulation heterogeneity in human acute myeloid leukemia determined by monoclonal antibodies. Blood 64:275, 1984

24. Bradstock KF, Favaloro EJ, Kabral A, Kerr A, Hughes WG, Musgrove E: Myeloid progenitor surface antigen identified by monoclonal antibody. Br J Haematol 61:11, 1985

25. Browman GP, Neame PB, Soamboonsrup P: The contribution of cytochemistry and immunophenotyping to the reproducibility of the FAB classification in acute leukemia. Blood 68:900, 1986

26. Neame PB, Soamboonsrup P, Browman G, Barr RD, Saeed N, Chan B, Pai M, Benger A, Wilson WEC, Walker IR, McBride JA: Simultaneous or sequential expression of lymphoid and myeloid phenotypes in acute leukemia. Blood 65:142, 1985

27. Koziner B, Gebhard D, Denny T, McKenzie S, Clarkson BD, Miller DA, Evans RL: Analysis of T cell differentiation antigens in acute lymphatic leukemia using monoclonal antibodies Blood 60:752, 1982

28. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human intrathymic differentiation. Analysis of normal thymocytes and leukemic lymphoblasts of T cell lineage Proc Natl Acad Sci USA 77:1588, 1980

29 Sobol RE, Royston I, LeBien TW, Minowada J, Anderson K, Davey FR, Cuttner J, Schiffer C, Ellison RR, Bloomfield CD: Adult acute lymphoblastic leukemia phenotypes defined by monoclonal antibodies. Blood 65:730, 1985

30. Newman RA, Greaves MF: Characterization of HLA-DR antigens on leukemic cells. Clin Exp Immunol 50:41, 1982

31. Hanjan SNS, Kearney JF, Cooper MD. A monoclonal antibody (MMA) that identifies a differentiation antigen on human myelomonocytic cells. Clin Immunol Immunopathol 23:172, 1982

32 Griffin JD, Linch D, Sabbath K, Larcom P, Schlossman SF. A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8:521, 1984

33. Todd RF, Nadler LM, Schlossman SF: Antigens of human monocytes identified by monoclonal antibodies. J Immunol 126:1435, 1981

34 Zola H, McNamara P, Thomas M, Smart IJ, Bradley J: The preparation and properties of monoclonal antibodies against human granulocyte membrane antigens. Br J Haematol 48:481, 1981

35. Thurlow PJ, Barlow B, Connellan JM, McKenzie IFC. Detection of glycoprotein IIb and IIIa by monoclonal antibodies. Br J Haematol 55:123, 1983

36. Koike T: Megakaryoblastic leukemia: The characterization and identification of megakaryoblasts Blood 64:683, 1984

37. Todd RF, Schlossman SF: Analysis of antigenic determinants on human monocytes and macrophages. Blood 59:775, 1982

38 Huang M-J, Li C-Y, Nichols WL, Young J-H, Katzmann JA: Acute leukaemia with megakaryocytic differentiation. A study of 12 cases identified immunocytochemically. Blood 64:427, 1984

39. Bollum FJ: Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 54:1203, 1979

40. Levy R, Warnke R, Dorfman RF, Haimovich J: The monoclonality of human B cell lymphomas. J Exp Med 145:1014, 1977

41. Han T, Ozer H, Bloom M, Sagawa K, Minowada J: The presence of monoclonal cytoplasmic immunoglobulin in leukemic B cells from patients with chronic lymphocytic leukemia. Blood 59:435, 1982

42. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: The French-American-British (FAB) Co-operative Group: Proposals for the classification of the myelodysplastic syndromes Br J Haematol 51:189, 1982

43. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: (FAB Co-Op Group): A variant form of hypergranular promyelocytic leukaemia (M3). Ann Intern Med 92:261, 1980 (letter)

44 Mirro J, Antoun GR, Zipf TF, Melvin S, Stass S: The E

rosette–associated antigen of T cells can be identified on blasts from patients with acute myeloblastic leukemia. Blood 65:363, 1985

45. Neame PB, Soamboonsrup P E-rosette formation in acute nonlymphocytic leukemia. Blood 66:247, 1985 (letter)

46. Mirro J, Zipf TF, Pui C-H, Kitchingman G, Williams D, Melvin S, Murphy SB, Stass S· Acute mixed lineage leukemia Clinicopathologic correlations and prognostic significance. Blood 66 1115, 1985

47. Greaves MF, Chan LC, Furley AJW, Watt SM, Molgaard HV. Lineage promiscuity in hemopoietic differentiation and leukemia. Blood 67:1, 1986

48. Neame PB, Soamboonsrup P, Browman GP: Immunological phenotyping of acute leukemia. Blood 66 180a, 1985 (Suppl. 1) (abstr)

49. Griffin JD, Todd RF, Tirz J, Nadler LM, Canellos GP, Rosenthal D, Gallivan M, Beveridge RP, Weinstein H, Karp K, Schlossman SF: Differentiation patterns in the blastic phase of chronic myeloid leukemia. Blood 61:85, 1983

50. Koziner B, Mertelmann R, Andreeff M, Arlin Z, Hansen H, De Harven E, McKenzie S, Gee T, Good RA, Clarkson B. Heterogeneity of cell lineages in L3 leukemias. Blood 55:694, 1980

51. Thiel E: Cell surface markers in leukemia: Biological and clinical correlations. CRC Crit Rev Oncol Hematol 2·209, 1985

52. Greaves MF, Janossy G, Peto J, Kay H: Immunologically defined subclasses of acute lymphoblastic leukaemia in children: their relationship to presentation features and prognosis. Br J Haematol 48:179, 1981

53. Peiper SC, Stass SA. Markers of cellular differentiation in acute lymphoblastic leukemia. Arch Pathol Lab Med 106:3, 1982

54. Blazar BR, Brunning RD, Bloomfield CD, McKenna RW, Robison LL, LeBien TW, Gajl-Peczalska K, Nesbit ME, Kersey JH· Correlation of monoclonal antibody (MoAb) phenotypes with FAB morphology in acute nonlymphocytic leukemia (ANLL) Blood 60·120a, 1982 (Suppl 1) (abstr)

55. Eridani S, Chan LC, Halil O, Pearson TC: Acute biphenotypic leukaemia (myeloid and Null ALL type) supervening in a myelodysplastic syndrome. Br J Haematol 61:525, 1985

56. Catovsky D, DeCastro JT: The classification of acute leukaemia (AL) and its clinical significance. Schweiz Med Wochenschr 113.1434, 1983

57. Bettelheim P, Paietta E, Majdic O, Gadner H, Schwarzmeier J, Knapp W Expression of a myeloid marker on TdT-positive acute lymphocytic leukemia cells. Evidence of double-fluorescence staining. Blood 60:1392, 1982

58. Paietta E, Bettelheim P, Schwarzmeier JD, Lutz D, Majdic O, Knapp W: Distinct lymphoblastic and myeloblastic populations in TDT positive acute myeloblastic leukemia: Evidence by double-fluorescence staining. Leuk Res 7·301, 1983

59. Bernard A, Bernstein I, Boumsell L, Dausset J, Evans R, Hansen J, Haynes B, Kersey J, Knapp W, McMichael A, Milstein C, Reingerz E, Ritts RE, Schlossman SF (IUIS - WHO Nomenclature Subcommittee): Nomenclature. J Immunol 134:659, 1985

60. Dinndorf PA, Andrews RG, Benjamin D, Ridgway D, Wolff L, Bernstein ID Expression of normal myeloid-associated antigens by acute leukemia cells Blood 67·1048, 1986

61. Foon KA, Todd RF: Immunologic classification of leukemia and lymphoma. Review Blood 68:1, 1986

62. Crist W, Boyett J, Roper M, Pullen J, Metzgar R, van Eys J, Ragab A, Starling K, Vietti T, Cooper M Pre-B cell leukemia responds poorly to treatment A Pediatric Oncology Group study. Blood 63:407, 1984

63. Sallam SE, Ritz J, Pesando J, Gelber R, O'Brien C, Hitchcock S, Coral F, Schlossman SF: Cell surface antigens: Prognostic implications in childhood acute lymphoblastic leukemia. Blood 55 395, 1980

64 Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 71:301, 1983

65 Arnold A, Cossman J, Bakhshi A, Jaffe ES, Waldmann TA, Korsmeyer SJ: Immunoglobulin-gene rearrangement as unique clonal markers in human lymphoid neoplasms. N Engl J Med 309 1593, 1983

66. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW: A human T cell–specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature 308·145, 1984

67 Hendrick SM, Nielsen EA, Kavaler J, Cohen DI, Davis MM: Sequence relationship between putative T cell receptor polypeptides and immunoglobulins. Nature 308:153, 1984

68. Yanagi Y, Chan A, Chin B, Minden M, Mak TW· Analysis of cDNA clones specific for human T cells and the α and β chains of the T cell receptor heterodimer from a human T cell line. Proc Natl Acad Sci USA 82:3430, 1985

69. Minden MD, Toyonaga B, Ha K, Yanagi Y, Chin B, Gelfand E, Mak T: Somatic rearrangement of T cell antigen receptor gene in human T cell malignancies. Proc Natl Acad Sci USA 82 1224, 1985

70 Rovigatti U, Mirro J, Kitchingman G, Dahl G, Ochs J, Murphy S, Stass S Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia Blood 63:1023, 1985

71 Reinherz EL, Haynes BP, Nadler LM, Bernstein ID (ed): Leukocyte Typing II (Vols 1, 2 and 3). New York, Springer Verlag, 1986

72 Linch DC, Griffin JD· Monoclonal antibodies reactive with myeloid associated antibodies, in Beverley PCL (ed): Monoclonal Antibodies. New York, Churchill Livingstone, 1986 p 222

73. Janossy G, Bollum FJ, Campana D. Immunofluorescent studies in leukemia diagnosis, in Beverley PCL (ed): Monoclonal Antibodies. New York, Churchill Livingstone, 1986, p 97

Decreased Incidence of Marrow Graft Rejection in Patients With Severe Aplastic Anemia: Changing Impact of Risk Factors

By H. Joachim Deeg, Steven Self, Rainer Storb, Kristine Doney, Frederick R. Appelbaum, Robert P. Witherspoon, Keith M. Sullivan, Karen Sheehan, Jean Sanders, Eric Mickelson, and E. Donnall Thomas

Patients with severe aplastic anemia were conditioned with cyclophosphamide (200 mg/kg) and given marrow grafts from HLA-identical family members. Among 233 patients transplanted, 225 survived ≥14 days and could be evaluated for engraftment. Forty-four of the 225 rejected their graft; 33 of these died and 11 survive. One hundred eighty-one patients had sustained engraftment; of these, 46 died and 135 survived. Binary logistic regression analyses revealed five risk factors for graft rejection: (a) year of transplant, (b) a large number of platelet transfusions, (c) a positive relative response in mixed leukocyte culture, (d) a low marrow cell dose, and (e) omission of donor buffy coat cell infusion for transfused patients. These data show that patients transplanted recently had a lower likelihood of graft rejection than did patients transplanted in earlier years. Conceivably, this was related to changes in transfusion practices, but other factors as yet unidentified are likely to be involved. The data confirm that the largest possible number of marrow cells should be transplanted. Although the difference in the incidence of graft rejection between untransfused and transfused patients was not significant, it should be noted that transfused patients were given buffy coat cells. Because the addition of buffy coat cells results in a higher incidence of chronic graft-*v*-host disease (GVHD), it is still desirable to transplant patients with marrow alone early in their course before they have been transfused.

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MARROW TRANSPLANTATION is an effective treatment for patients with severe aplastic anemia. While initially 25% to 45% of patients became long-term survivors,[1–5] more recent reports indicate that 60% to 80% of patients with aplastic anemia are cured by allogeneic marrow transplantation.[6–12] To a great extent, this progress is the result of a reduction in the incidence of marrow graft rejection from 30% to 60% down to 15%, due to the use of donor buffy coat cells or more intensive preparative regimens. During the past 4 to 5 years, we noticed a further decrease in the incidence of marrow graft rejection in transfused patients, to ~5%. This change occurred even though we continued to use our standard regimen of cyclophosphamide and postgrafting immunosuppression, consisting of methotrexate either alone or combined with cyclosporine. This observation prompted us to reexamine factors potentially associated with marrow graft rejection.

MATERIALS AND METHODS

From October 1970 through December 1984, 233 patients with aplastic anemia referred to the Fred Hutchinson Cancer Research Center were conditioned with cyclophosphamide and received a marrow transplant from an HLA-identical family member. Eight patients died <14 days after transplant from infections or heart failure[13] They could not be evaluated for engraftment and are excluded from the present analysis. Patient and donor characteristics on the remaining 225 patients included in the analysis are summarized in Table 1 HLA identity of marrow donor and patient was determined by serologic typing for HLA-A, B, and C, and by nonreactivity of donor and patient lymphocytes in mixed leukocyte culture (MLC) The response of patient cells to donor cells in MLC was compared with the response of patient cells to cells from unrelated donors and expressed as a relative response (RR). The average RR in a control group of 212 healthy HLA-identical siblings was 0.0 ± 2.6% (mean ± 2 SD). On this basis, a positive response was defined as an RR of >2.6%.[14]

Patients were conditioned with cyclophosphamide, 50 mg/kg/day for 4 consecutive days.[15] The 49 previously untransfused patients were given marrow only. Among 176 transfused patients, 90 were given marrow only, and 86 were given marrow plus viable donor buffy-coat cells. Initially, buffy coat cells were given to patients with positive in vitro sensitization tests.[16] Subsequently, all previously transfused patients were given buffy coat cells, regardless of in vitro test results.[16]

For graft-*v*-host disease (GVHD) prophylaxis, 186 patients were given intermittent methotrexate for 3 months,[17] 13 were given daily cyclosporine starting on day −1, continued in gradually reduced doses for 6 months[8,18]; 25 were given the same regimen of cyclosporine plus four doses of methotrexate on days 1, 3, 6, and 11 after transplant.[6,19]

Patients were monitored for evidence of engraftment or graft rejection by daily peripheral blood cell counts, weekly determinations of marrow cellularity, and intermittent monitoring of genetic markers such as cytogenetic analysis of peripheral blood and marrow cells, RBC antigen and enzyme phenotypes, and immunoglobulin allotypes.[13,16]

Informed consent was obtained from all patients. All marrow transplant protocols were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Analyses of graft rejection rates considered 20 factors, as listed in Table 2. Standard contingency table methods and binary logistic regression methods were used[20] The binary logistic regression method allowed several potential prognostic variables to be related simultaneously to graft rejection. Specifically, the odds for graft rejection (probability of graft rejection/1 minus probability of graft

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Table 1. Patient and Donor Characteristics

Datum	Value
Number of patients studied	225
Age in years, range (median)	2-60 (19)
25th percentile	13
75th percentile	27
Sex (M/F)	126/96
Etiology of aplastic anemia	
Idiopathic	159
Chemical	20
Hepatitis	16
Drugs	15
Fanconi's	7
Paroxysmal nocturnal hemoglobinuria	4
Pregnancy	4
Duration of disease (mo)	
Range (median)	0–224 (1 6)
Previously transfused	176
Untransfused	49
Previous therapy with	
Androgens	98
Steroids	115
Refractory to random donor platelets	60
Relative response (%)*	
Positive (>2.6)	25
Negative (≤2 6)	185
Donor/patient sex	
M/M	69
M/F	43
F/M	60
F/F	53
Donor/patient ABO	
Match	168
Mismatch	57
Donor buffy coat infusion	86
Marrow cell dose ($\times 10^{-8}$/kg), range (median)	0 61–15.5 (3.2)

Number of patients, unless indicated otherwise.

*In 15 patients, this information was not available

rejection) were expressed as a product of a baseline rate multiplied by a factor for each of the predictor variables included in the analysis. The method produced maximum likelihood estimates of the odds ratio associated with each variable. These estimates compared the odds of graft rejection associated with any specified level of a variable with the odds at a base level (specified by the data analyst). The method also produced tests of the hypothesis that the odds ratio function for each variable was equal to unity (ie, the hypothesis of no association between the variable and graft rejection after "accommodating" the other variable in the analysis). The possibility that the odds ratio for one variable may depend on the level of the second variable could be accommodated either by adding product terms to the regression equation or by restricting the analysis to patients with specified levels for the second variable. Asymptotic likelihood methods were used to calculate SE and significance levels.

RESULTS

Graft rejection and survival Forty-four patients rejected their grafts, and 181 had sustained engraftment. Graft rejection was the most important factor determining long-term survival: 73% for patients with engraftment and 27% for patients with graft rejection (Fig 1). All patients with engraftment have remained complete chimeras, as determined by blood genetic markers. Among 11 patients who survived after rejection, 4 showed recovery of autologous marrow function, and 7 had successful second marrow grafts.

Analysis of previously recognized risk factors. There was a decrease in the incidence of rejection in virtually every category of every risk factor examined; still, any transfusion prior to transplantation was associated with increased rejection (Table 2). There was a positive correlation between the number of units of platelets or red blood cells transfused and the probability of graft rejection. Other factors had a similar impact to that described previously (Table 2).

Year of transplant. In addition to previously recognized factors, the year of transplant had a significant effect, ie, patients transplanted in recent years had a lower incidence of rejection than did patients transplanted in earlier years. This effect of year of transplant was not explained by secular trends of previously identified risk factors. Therefore, we analyzed further the factors for which the initial analysis suggested some changes: transfusion and transfusion products.

Analysis of transfusion products. Among patients classified as transfused, there was a shift toward fewer transfusions of platelets and RBCs (Table 3). A test for association between year of transplant and number of transfusions (fewer transfusions in recent years) was significant for platelets ($P = .02$) but not for RBCs ($P = .2$). Some patients had been transfused on more than eight or ten occasions, but the exact number of transfusions could not be determined. They were included as "multiple" in the group given ≥40 U. We also examined whether the type of RBC transfusions given changed over time. For this purpose, we reviewed all patient charts to determine whether pretransplant patients had been transfused with whole blood, packed RBCs, frozen/washed RBCs, or leukofiltered RBCs. There was a trend toward fewer transfusions of any type in recent years, and no significant shift in favor of one transfusion product was observed (data not shown). Further analyses were impractical since patients usually had recived more than one type of transfusion product.

Binary logistic regression analyses. We then carried out binary logistic regression analyses to separate those factors that predicted graft rejection from those for which an association might exist on the basis of other characteristics. Results are summarized in Table 4. Analyses were carried out for all patients on whom data on relative responses in MLC were available, and separately in transfused patients and in the subgroups of transfused patients not given buffy coat cells. There was an inverse correlation between the number of marrow cells and the incidence of rejection for all three patient populations, and the infusion of buffy coat cells had a positive effect The relative response of lymphocytes in MLC was significantly associated with graft rejection for all patients. Donor sex, previously found to be a significant factor, was not significant.

The incidence of rejection among untransfused patients was similar to that reported previously,[21] but the incidence among transfused patients had decreased from 26% to 11% and was no longer significantly different from that in untransfused patients

Table 2 Risk Factors and Incidence of Marrow Graft Rejection in Univariate Analysis

Factor*	Category	Patients with Rejection/ Patients Studied (%)†	*P* Value‡
Year of transplant	<1975	11/39 (28)	
	1975–1977	19/69 (28)	
	1978–1980	7/52 (14)	
	1981–1984	7/65 (11)	<0.01
Transfused	Yes	39/176 (22)	
	No	5/49 (10)	0.09
Platelet transfusions (no. of units)	0	8/55 (14)	
	1–4	2/12 (17)	
	5–9	4/31 (13)	
	10–19	5/32 (16)	
	20–39	2/23 (9)	
	≥40 or multiple§	23/72 (31)	0.02
RBC transfusion (no. of units)	0	5/46 (11)	
	1–4	9/69 (13)	
	5–9	6/38 (16)	
	10–19	9/27 (33)	
	20–39	5/17 (29)	
	≥40 or multiple	10/28 (37)	<0.01
Refractory to random donor platelet transfusions	Yes	17/60 (28)	
	No	27/164 (16)	0.03
	Unknown	0/1 (0)	
Treatment with androgens	Yes	25/98 (26)	
	No	19/127 (15)	0.02
LAF room	Yes	11/81 (14)	
	No	33/144 (23)	0.03
Patient ABO group	A	20/70 (29)	
	B	7/28 (25)	
	AB	0/8 (0)	
	O	17/119 (14)	0.04
Relative response (%)	<2.6	25/185 (14)	
	≥2.6	15/25 (60)	<0.001
Bone marrow cell dose ($\times 10^{-8}$/kg)	<2	10/32 (31)	
	2–2.9	15/66 (23)	
	3–3.9	9/56 (16)	
	4–5.9	9/47 (19)	
	≥6.0	1/24 (4)	0.08
Buffy coat cells	Yes	8/86 (9)	
	No	36/139 (26)	<0.01
GVHD prophylaxis	Methotrexate (MTX)	40/186 (21)	
	Cyclosporine (CSP)	1/13 (8)	
	MTX + CSP	3/25 (12)	0.8

LAF, laminar air flow.

*Donor/patient sex combination, patient age, donor ABO group, donor/patient ABO mismatch, interval from diagnosis to transplant, previous steroid therapy, etiology of aplastic anemia, and type of RBC transfusion used (details in Table 4) were not significant.

†In some patients, the relevant information was not available; therefore, the numbers in each group do not total 40/225.

‡Significance levels are based on uncorrected chi-square statistics for 2 × K contingency tables.

§Multiple. patients were multiply transfused, but the exact number of transfusions was not known.

Among transfused patients there was a positive correlation between the amount of platelets transfused and the probability of graft rejection. Patients who had received <40 U (ie, five to six transfusions) had an incidence of rejection significantly lower than that of patients given more transfusions.

The year of transplantation had an impact in all patient groups and was most significant in transfused patients Patients transplanted since 1980 had an incidence of rejection significantly lower than that of patients transplanted earlier, even after simultaneous adjustment was made for other risk factors such as number of platelet transfusions and infusion of buffy coat cells. Other factors for which univariate analysis (Table 2) had suggested a significant effect were no longer significant.

DISCUSSION

The current analysis was prompted by the observation in recent years of a markedly decreased incidence of marrow graft rejection in patients with severe aplastic anemia.[6] Statistical analysis identified year of transplant as an independent "risk factor" for graft rejection. No clear explanation for this secular trend was apparent. In contrast to

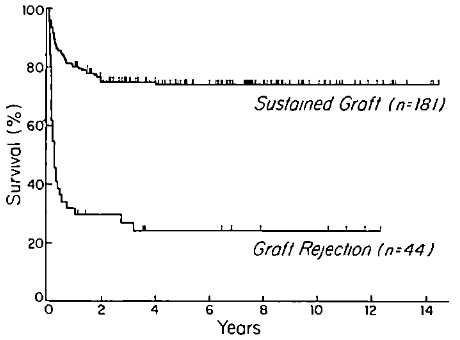

Fig 1. Kaplan-Meier product limit estimates[39] of survival among patients with severe aplastic anemia conditioned with cyclophosphamide and given a marrow graft from an HLA-identical family member. Shown are data on 181 patients with sustained engraftment and 44 who rejected their first graft (survival as of July 1, 1985).

suggestions made by others,[8,22] the use of postgrafting cyclosporine had no significant effect on sustained engraftment, which was similar in patients given a standard regimen of methotrexate after grafting.[6] Pregrafting transfusion per se was no longer a significant risk factor. Indeed, in recent years, graft rejection in transfused patients overall, albeit with the addition of buffy coat cells, has declined to the same level as that observed in untransfused patients.[21] The probability of rejection was significantly increased in patients given large numbers of platelet transfusions, however.

Although direct proof is difficult to provide, the decrease in graft rejection may have been related to changes in transfusion practices We showed in a canine model of DLA-identical littermates that transfusion of leukocyte-depleted platelets or RBCs, rather than whole blood, decreased the probability of sensitization and subsequent marrow graft rejection.[23,24] An involvement of donor leukocytes in recipient sensitization has also been suggested by human studies on platelet refractoriness.[26,27] Furthermore, pretransplant transfusions from family members are now avoided.[27] Whole blood transfusions are not commonly used; frequently, leukocyte-poor preparations, sometimes obtained from single donors, are substituted. In addition, longer storage times for RBCs and platelets have been adopted in recent years. The number and stimulating ability of leukocytes in transfusion products are known to decline with time.[27,28] Thus, these approaches may decrease the incidence of sensitization, especially against the marrow donor, in the transfusion recipient. Consequently, the incidence of rejection would be similar in transfused and untransfused patients The observation that year of transplant was most significant for transfused patients but less so for untransfused patients was also in agreement with this probability (Table 4).

The antigens responsible for sensitization and graft rejection remain to be determined. Whether platelet-associated antigens are involved is not known. Differences in RBC antigens between donor and recipient have generally not been found to be associated with marrow graft rejection.[29-31] Results in dogs suggest that at least two polymorphic non-DLA antigen systems expressed on leukocytes are involved.[23] The relevant antigens[23] appear to be expressed on cells with accessory function in vitro[24,32] and possibly in vivo.[33] Cells inducing sensitization in vivo may be those that stimulate the increased response of host cells in MLC in vitro In apparent agreement with this reasoning was the observation that in recent years very few transfused patients had a positive relative response as compared with an incidence of 20% to 30% in earlier years.[13,34]

Beyond the influence of transplant year and number of platelet transfusions, the present analysis confirmed three previously identified risk factors.[34] The significance of a positive relative response in MLC probably cannot be completely separated from the effect of pretransplant transfusion and has been discussed above. The effect of marrow cell dose, although confirmed, was less prominent than in previous analyses.[13,34] This may be due in part to a statistical artifact. Because marrow cell dose was initially identified as a risk factor by an exploratory analysis, some decline of its effect over time should be expected.

Previous studies have shown that patients given buffy coat in addition to marrow cells rejected their grafts less frequently (14%) than did those given marrow only (32%),[16] a finding confirmed in the present analysis Patients not given buffy coat cells had a risk of graft rejection more than twofold higher than that of patients who received buffy coat

Table 3. Distribution of Numbers of Units of Transfused Platelets and RBCs by Year of Transplant Among Transfused Patients

		Year of Transplant			
		<1975	1975–1977	1978–1980	≥ = 1981
Transfusion Product	No of Units	No of Patients			
Platelets	0–4	2	6	5	12
	5–9	6	5	4	11
	10–19	8	7	8	8
	20–39	9	5	5	4
	≥40 or multiple*	12	33	8	18
Red blood cells	0–4	12	16	13	25
	5–9	8	12	5	13
	10–19	9	6	5	7
	20–39	3	10	3	1
	≥40 or multiple	5	12	4	7

*In several multiply transfused patients, the exact number of transfusions given could not be determined.

Table 4. Binary Logistic Regression Analyses of Factors Associated With Graft Rejection in 210 Patients

Covariate*	Odds Ratio Estimate		
	All Patients (n = 210)†	Transfused Patients (n = 165)‡	Transfused Patients Without Buffy Coat (n = 86)§
Marrow cell dose ($\times 10^{-8}$/kg)‖	0.78 (.04)¶	0.77 (.04)	0.70 (.04)
Relative response (0 = negative, 1 = positive)	5.83 (<.01)	6.81 (<.01)	9.08 (<.01)
Buffy coat cells (0 = yes, 1 = no)	2.69 (.03)	2.35 (.08)	—
Platelet units (0 = <40, 1 = ≥40 or multiple)	2.38 (.05)	2.44 (.05)	2.70 (.08)
Year of transplant (0 = transplant year ≤1980, 1 = tranplant year >1980)	0.43 (.07)	0.31 (.03)	0.39 (.18)
Untransfused (0 = no, 1 = yes)	0.64 (0.44)	—	—

*Other covariates entered into the regression analysis but found not significant at the 5% level were patient age, patient sex, donor sex, untransfused, RBC units, laminar air flow room, prior treatment with androgens, refractory to random donor platelets, months from diagnosis to transplantation, ABO mismatch, patient ABO, and interactions between year of transplant and all other covariates in the model.

†Two hundred ten patients with known relative response, 40 of whom rejected their graft.

‡One hundred sixty-five patients with known relative response, 35 of whom rejected their graft.

§Eighty-six patients with known relative response who received buffy coat cells; 27 rejected their graft.

‖The odds of rejection were estimated to decline by a factor of 0.78 with each increase in marrow cell dose of 1 × 10^8/kg.

¶Significance level for testing odds ratio = 1.

cells. Indeed, our analysis suggests that the beneficial effect of buffy coat cells has increased over time. In a recent analysis limited to pediatric patients (aged <17 years), the addition of buffy coat cells was not associated with decrease in rejection as compared with rejection in patients given marrow only.[35] This question requires further study, especially since the infusion of buffy coat cells is associated with an increased incidence of chronic GVHD.[36]

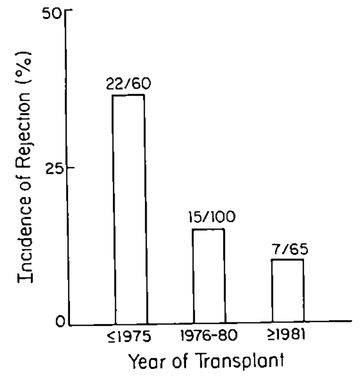

Fig 2. Decline of the incidence of graft rejection over time. At the top of each column the number of patients rejecting/total number of patients transplanted is shown.

The influence of donor sex on engraftment has been controversial. We found previously that grafts from male donors were rejected more frequently than were grafts from female donors[13]; however, the opposite association was reported by the International Marrow Transplant Registry and the European Bone Marrow Transplant Group.[2,37] The present finding of no effect of donor sex on engraftment is in agreement with yet another report by the European Marrow Transplant Group.[38] Again, one should keep in mind that some of the associations described above were the products of exploratory data analyses including a number of additional potentially predictive factors. Therefore, corresponding significance levels must be interpreted conservatively.

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REFERENCES

1. Storb R, Thomas ED, Buckner CD, Appelbaum FR, Clift RA, Deeg HJ, Doney K, Hansen JA, Prentice RL, Sanders JE, Stewart P, Sullivan KM, Witherspoon RP: Marrow transplantation for aplastic anemia. Semin Hematol 21:27, 1984

2. Bortin MM, Gale RP, Rimm AA, for the Advisory Committee of the International Bone Marrow Transplant Registry: Allogeneic bone marrow transplantation for 144 patients with severe aplastic anemia. JAMA 245:1132, 1981

3. Elfenbein GJ, Mellits ED, Santos GW, for the Johns Hopkins Bone Marrow Transplant Program. Patients with aplastic anemia: Engraftment and survival after allogeneic bone marrow transplantation for severe aplastic anemia. Transplant Proc 15:1412, 1983

4. Bacigalupo A, DiGiorgio F, Congiu M, van Lint MT, Frassoni F, Daga MG, Gogioso L, Marmont AM: Treatment of severe aplastic anemia in Europe 1970–1983: A report of the EBMT SAA Working Party Exp Hematol 13:56, 1985 (suppl 17)

5. Camitta BM, Storb R, Thomas ED: Aplastic anemia: Pathogenesis, diagnosis, treatment, and prognosis. N Engl J Med 306:645, 712, 1982

6. Storb R, Deeg HJ, Farewell V, Doney K, Appelbaum F, Beatty P, Bensinger W, Buckner CD, Clift R, Hansen J, Hill R, Longton G, Lum L, Martin P, McGuffin R, Sanders J, Singer J, Stewart P, Sullivan K, Witherspoon R, Thomas ED: Marrow transplantation for severe aplastic anemia: Methotrexate alone compared to a

combination of methotrexate and cyclosporine for prevention of acute graft-versus-host disease. Blood 68.119, 1986

7. Deeg HJ, Shulman HM, Schmidt E, Yee GC, Thomas ED, Storb R Marrow graft rejection and veno-occlusive disease of the liver in patients with aplastic anemia conditioned with cyclophosphamide and cyclosporine. Transplantation (in press)

8 Hows JM, Palmer S, Gordon-Smith EC· Cyclosporine and graft failure following bone marrow transplantation for severe aplastic anemia Br J Haematol 60·611, 1985

9. Feig SA, Champlin R, Arenson E, Yale C, Ho W, Tesler A, Gale RP, for the UCLA Bone Marrow Transplant Team. Improved survival following bone marrow transplantation for aplastic anaemia Br J Haematol 9:509, 1983

10. Champlin RE, Feig SA, Gale RP. Case problems in bone marrow transplantation: I. Graft failure in apalstic anemia. Its biology and treatment. Exp Hematol 12:728, 1984

11. Ramsay NKC, Kim TH, McGlave P, Goldman A, Nesbit ME, Krivit W, Wood WG, Kersey JH. Total lymphoid irradiation and cyclophosphamide conditioning prior to bone marrow transplantation for patients with severe aplastic anemia Blood 62.622, 1983

12. Gluckman E, Devergie A, Benbunan A, Bridier A, Dutreix J. Bone marrow transplantation in severe aplastic anemia using cyclophosphamide and thoraco abdominal irradiation. Transplant Proc 15.2628, 1983

13 Storb R, Prentice RL, Thomas ED, Appelbaum FR, Deeg HJ, Doney K, Fefer A, Goodell BW, Mickelson E, Stewart P, Sullivan KM, Witherspoon RP· Factors associated with graft rejection after HLA-identical marrow transplantation for aplastic anaemia. Br J Haematol 55 573, 1983

14 Mickelson EM, Clift RA, Fefer A, Storb R, Thomas ED, Warren RP, Hansen JA. Studies of the response in mixed leukocyte culture of cells from patients with aplastic anemia to cells from HLA-identical siblings Transplantation 32 90, 1981

15. Storb R, Thomas ED, Buckner CD, Clift RA, Johnson FL, Fefer A, Glucksberg H, Giblett ER, Lerner KG, Neiman P· Allogeneic marrow grafting for treatment of aplastic anemia Blood 43:157, 1974

16. Storb R, Doney KC, Thomas ED, Appelbaum F, Buckner CD, Clift RA, Deeg HJ, Goodell BW, Hackman R, Hansen JA, Sanders J, Sullivan K, Weiden PL, Witherspoon RP: Marrow transplantation with or without donor buffy coat cells for 65 transfused aplastic anemia patients. Blood 59:236, 1982

17. Storb R, Epstein RB, Graham TC, Thomas ED. Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow grafts Transplantation 9:240, 1970

18 Deeg HJ, Storb R, Thomas ED, Flournoy N, Kennedy MS, Banaji M, Appelbaum FR, Bensinger WI, Buckner CD, Clift RA, Doney K, Fefer A, McGuffin R, Sanders JE, Singer J, Stewart P, Sullivan KM, Witherspoon RP: Cyclosporine as prophylaxis for graft-versus-host disease: A randomized study in patients undergoing marrow transplantation for acute nonlymphoblastic leukemia Blood 65:1325, 1985

19 Deeg HJ, Storb R, Appelbaum FR, Kennedy MS, Graham TC, Thomas ED: Combined immunosuppression with cyclosporine and methotrexate in dogs given bone marrow grafts from DLA-haploidentical littermates Transplantation 37.62, 1984

20 Cox DR· The Analysis of Binary Data London, Methuen, 1970

21. Anasetti C, Doney KC, Storb R, Meyers JD, Farewell VT, Buckner CD, Appelbaum FR, Sullivan KM, Clift RA, Deeg HJ, Fefer A, Martin PJ, Singer JW, Sanders JE, Stewart PS, Witherspoon RP, Thomas ED: Marrow transplantation for severe aplastic anemia Long term outcome in 50 "untransfused" patients. Ann Intern Med 104 461, 1986

22. Bacigalupo A, Frassoni F, van Lint MT, Raffo MR, Vitale V, Corbetta G, Chierichetti S, Marmont AM. Cyclosporin A in marrow transplantation for leukemia and aplastic anemia. Exp Hematol 13.244, 1985

23 Storb R, Weiden PL, Deeg HJ, Graham TC, Atkinson K, Slichter SJ, Thomas ED. Rejection of marrow from DLA-identical canine littermates given transfusions before grafting: Antigens involved are expressed on leukocytes and skin epithelial cells but not on platelets and red blood cells. Blood 54 477, 1979

24. Deeg HJ, Torok-Storb BJ, Storb R, Weiden PL, DeRose S, Graham TC, Atkinson K, Thomas ED. Rejection of DLA-identical canine littermate marrow after transfusion-induced sensitization: Antigens involved are expressed on cotton-wool adherent but not on nonadherent mononuclear cells, granulocytes, or thoracic duct lymphocytes, in Baum SJ, Ledney GD, Khan A (eds): Experimental Hematology Today Basel, Karger, 1981, p 31

25. Eernisse JG, Brand A: Prevention of platelet refractoriness due to HLA antibodies by administration of leukocyte-poor blood components Exp Hematol 9:77, 1981

26. Schiffer CA, Dutcher JP, Aisner J, Hogge DE, Wiernik PH, Reilly JP: A randomized trial leukocyte-depleted platelet transfusion to modify alloimmunization in patients with leukemia. Blood 62·815, 1983

27. Slichter SJ. Prevention of platelet alloimmunization, in Murawski K, Peetoom F (eds): Transfusion Medicine· Recent Technological Advances New York, Liss, 1986, p 83

28. Oh JH, McClure HM· Lymphocytotoxic antibodies induced by fresh blood, stored blood, and platelets in rhesus monkeys Transplant Proc 14:410, 1982

29 Bensinger WI, Buckner CD, Thomas ED, Clift RA. ABO-incompatible marrow transplants Transplantation 33:427, 1982

30. Braine HG, Sensenbrenner LL, Wright SK, Tutschka PJ, Saral R, Santos GW Bone marrow transplantation with major ABO blood group incompatibility using erythrocyte depletion of marrow prior to infusion. Blood 60:420, 1982

31 Hershko C, Gale RP, Ho W, Fitchen J. ABH antigens and bone marrow transplantation. Br J Haematol 44.65, 1980

32. Aprile JA, Deeg HJ· UV irradiation of canine dendritic cells prevents mitogen induced cluster formation and lymphocyte proliferation. Transplantation (in press)

33. Deeg HJ, Aprile J, Graham TC, Appelbaum FR, Storb R Ultraviolet irradiation of blood prevents transfusion-induced sensitization and marrow graft rejection in dogs Blood 67.537, 1986

34. Storb R, Prentice RL, Thomas ED. Marrow transplantation for treatment of aplastic anemia: An analysis of factors associated with graft rejection. N Engl J Med 296:61, 1977

35. Sanders JE, Whitehead J, Storb R, Buckner CD, Clift RA, Mickelson E, Appelbaum FR, Bensinger WI, Stewart PS, Doney K, Sullivan K, Deeg HJ, Witherspoon RP, Thomas ED: Marrow transplant experience for children with aplastic anemia Pediatrics 77:179, 1986

36 Storb R, Prentice RL, Sullivan KM, Shulman HM, Deeg HJ, Doney KC, Buckner CD, Clift RA, Witherspoon RP, Appelbaum FA, Sanders JE, Stewart PS, Thomas ED: Predictive factors in chronic graft-versus-host disease in patients with aplastic anemia treated by marrow transplantation from HLA-identical siblings. Ann Intern Med 98·461, 1983

37 Gluckman E, Barrett AJ, Arcese W, Devergie A, Degoulet P: Bone marrow transplantation in severe aplastic anaemia· A survey of the European Group for Bone Marrow Transplantation. Br J Haematol 49.165, 1981

38 Gluckman E· Report of the EGBMT working party on severe aplastic anemia. Exp Hematol 10:14, 1982 (suppl 10)

39 Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53·457, 1958

bcr Rearrangement and Translocation of the c-*abl* Oncogene in Philadelphia Positive Acute Lymphoblastic Leukemia

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The Philadelphia (Ph^1) chromosome, the cytogenetic hallmark of chronic myeloid leukemia (CML), has also been detected in a significant number of acute lymphoblastic leukemias (ALL). Using in situ hybridization, we demonstrate that in accordance with observations in CML the Ph^1 chromosome in ALL patients is the result of a consistent translocation of the c-*abl* oncogene to the Ph^1 chromosome. Southern blot analysis using *bcr* probes, however, suggests that Ph^1-positive ALL includes heterogeneous leukemic subtypes: six ALL patients showed *bcr* rearrangements as observed in CML; in three other patients recombination involving 5′ *bcr* sequences could be demonstrated, but the corresponding translocated 3′ *bcr* sequences were not detectable. A third group of five patients did not show any *bcr* rearrangements at all. Northern blot analysis using RNA from three Ph^1-positive ALL patients revealed that in the leukemic cells of two patients larger c-*abl* mRNA transcripts were present, as in CML. In the RNA of one patient without a detectable *bcr* rearrangement, only the normal c-*abl* mRNA transcripts are present. The observed heterogeneity in *bcr* rearrangements of this group of Ph^1-positive ALL patients is in contrast with the consistent results obtained in more than 50 Ph^1-positive CML patients investigated in chronic and acute states.

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THE PHILADELPHIA (Ph^1) chromosome, an abnormal chromosome 22, is strongly associated with one type of human leukemia, chronic myeloid leukemia (CML).[1,2] It is found in the leukemic cells of more than 90% of all CML patients, both in the chronic and acute (blast crisis) phase of the disease.[3] The Ph^1 chromosome has also been reported in other malignant hematopoietic disorders,[4] including different subtypes of acute leukemias in which no preceding chronic phase has been observed.[5-8] The Ph^1 chromosome is found in the leukemic cells of 2% to 3% of the patients presenting with acute myeloid leukemia (AML) and a similar incidence (2% to 6%) is reported for childhood acute lymphoblastic leukemia (ALL).[9-10] In adult ALL the Ph^1 chromosome is the most frequent chromosomal abnormality with an incidence of 17% to 25%.[3,11] The clinical distinction between blast crisis of CML and de novo Ph^1-positive acute leukemia is not always clear.[5,12,13] However, at the time of blast crisis the majority of the CML patients exhibit a karyotypic evolution characterized by additional nonrandom chromosomal aberrations such as trisomy 8 and 19, a second Ph^1 or isochromosome 17q.[3] Furthermore, the presence of Ph^1-negative cells in the bone marrow during the acute phase and the elimination of Ph^1-positive cells from the bone marrow during remission are typical features of cases presenting as Ph^1-positive acute leukemias with no known prior CML.

In CML as well as in acute leukemias, the Ph^1 chromosome usually results from a translocation between chromosome 9 and 22t(9;22)(q34;q11). Previous studies using the leukemic cells of Ph^1-positive CML patients indicated that in CML this translocation is reciprocal.[14] A consistent translocation of the human c-*abl* oncogene, normally located on chromosome 9q34, to the Ph^1 chromosome was observed in standard and variant translocations.[15] In all these Ph^1-positive CML patients the c-*abl* oncogene was translocated to a specific, limited area on chromosome 22, the breakpoint cluster region (*bcr*).[16] Recently we have established that *bcr* is part of a gene[17] and as a consequence of the Ph^1-translocation the "bcr" gene located in this area is disrupted and c-*abl* sequences are linked to the 5′ *bcr* sequences.[18] Transcription of this DNA segment results in a new 8.5-kb mRNA species, which contains 5′ *bcr* and 3′ c-*abl* sequences [19-23]

Since the Ph^1 chromosomes of CML and acute leukemias are cytogenetically indistinguishable, it was of interest to investigate whether the described molecular aspects of the Ph^1 chromosome in CML are also present in Ph^1-positive acute leukemia. Here we report studies on the Ph^1 chromosome in ALL patients which demonstrate that similar molecular characteristics can be found in some but not all Ph^1-positive ALL patients.

MATERIAL AND METHODS

Patients We investigated the leukemic cells of 22 ALL patients referred to the cytogenetic units at Rotterdam (patients R1 through R7), Ulm (patients U1 through U6) and London (patients L1 through L9). The ALL diagnosis was based on clinical and hematologic data, bone marrow morphology, cytochemistry, and immunologic studies of the blast cells Patient R1, a 23-year-old female, was studied during complete remission of Ph^1-positive cALL. Cytogenetic studies revealed a normal karyotype (46,XX). Relevant clinical, hematologic, and cytogenetic data of all other patients are given in Tables 1, 2, and 3.

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Table 1. Ph^1-Negative ALL Patients

Patient No	Age/Sex (Years)	WBC 10^9/L	% Blast		Immunophenotype	Cytogenetics		Rearranged *bcr**
			BM	PB		Cells Analyzed	Karyotype	
L2	19 M	4.3	95	51	cALL	20	46, XY	—
L3	44 M	16.0	50	25	ALL†	20	46, XY	—
L4	67 F	60.6	80	78	T-ALL	20	46, XX	—
L5	36 M	70.4	99	89	cALL	20	46, XY	—
L6	25 M	15 1	99	57	cALL	50	46, XY	—
L7	17 M	21 7	95	69	cALL	8	46, XY	—

Abbreviations: BM, bone marrow; PB, peripheral blood, WBC, white blood cells.

*No *bcr* rearrangements.

†Unclassified ALL.

Table 2. Ph^1-Positive ALL Patients

Patient No	Age/Sex (Years)	Survival Week	WBC 10^9/L	% Blasts		Immunophenotype	Material	Cytogenetics		% Ph^1 Cells	Rearranged *bcr*
				BM	PB			Cells Analyzed	Karyotype*		
L8	19 F	44	22 7	90	90	cALL	BM	20	46,XX Ph^1	90	+
L9	52 F	28	121 0	84	86	cALL	BM	30	46,XX Ph^1	80	+
R2	3 M	67	88.2	87	89	cALL	PB	45	46,XY,t(9;22)(q34;q11) + add	91	−
R3	41 M	54	9.0	95	4 5	cALL	BM	80	46,XY,t(9;22)(q34;q11) + add	55	−
R4	19 M	46	3.6	99	45	cALL	BM‡	30	46,XY,t(14;22)(q32;q11)	83	−
R5	43 M	30	1 2	65	6	cALL	BM/PB	34	45,XY,t(9,22)(q34;q11) + add	70	−
U1	38 F	28	23 8	80	42	ALL§	PB	30	46,XX,t(14;22)(p22;q11)	30	+
U2	44 M	64	202 0	90	93	mixed AL‖	PB	31	46,XY,t(9;22)(q34;q11)	81	+
U3	39 M	48	129 0	50	15	mixed AL‖	BM	40	46,XY,t(5;9;22)(q13;q34;q11)	100	+
U4	31 M	12†	98 0	89	63	cALL	PB	30	46,XY,t(9;22)(q34;q11)	100	+
U5	21 M	12†	34 0	77	34	ALL§	BM	20	46,XY,t(9;22)(q34;q11)	100	ND

For abbreviations see Table 1; −, no *bcr* rearrangements; + with *bcr* rearrangements. ND, no data available.

*Ph^1 stands for 22q− without further information of karyotype changes.

The more complex karyotypes were as follows R2: 46,XY,t(9;22), t(3;9)(q26,p21), t(4;9q+)(p14,q21), t(5;17)(p14;q11); R3. 46,XY, t(9;22)(12.5%)/47,XY,t(9,22), +22q−(11%)/49,XY,t(9,22), +18, +21, +22q−(31.5%), R5 45,XY, t(1,9)(q25,p21), −8, 9p+q+ [8qter → q13: 9p21 → q34::22q11 → qter], 22q−.

†Was known as Ph^1-positive CML at age 9, achieved clinical remission and normal blood count until age 19.

‡Frozen BM from diagnostic phase after Ficoll.

§Unclassified ALL.

‖Both cALL-antigen− and various myeloid-antigen−positive cells.

Table 3. Ph^1-Positive ALL Patients Studied in Acute and Relapse Phases

Patient No	Age/Sex (Years)	Survival Weeks (w) Years (y)	WBC 10^9/L	% Blasts		Immunophenotype	Material	Cytogenetics		% Ph^1 (+) Cells	Rearranged *bcr*
				BM	PB			Cells Analyzed	Karyotype		
R6	61 F	56 w	209	75	60	cALL	BM D	38	46,XX,t(9;11;22)(q34;p15;q11)	60	+
			28.5	90	67	cALL	BM R*	57	idem/49,XXX,t(9,11;22),3p−,6q−, +7,+8p−,+21	51/32	+
U6	23 M	48 w	66.0	95	71	cALL	PB D	15	46,XY,t(9;22)(q34;q11)	53	+
			ND	ND	ND	cALL	PB R†	ND	ND	ND	+
L1	31 M	11 y	102	90	94	cALL‡	ND	ND	ND	ND	+
			17 5	2	0	CML	BM§	20	46,XY,Ph^1	100	ND
R7‖	50 F	156 w	33 7	61	71	cALL	BM	32	46,XX,t(9;14;22)(q33;q32;q11)	60	−
			89 8	3.2	4	CML	BM	21	46,XX,t(9;22)/46,XX,9q+,i(22q−)	70/30	+

Abbreviations: see Table 1 D, diagnostic; R, relapse, ND, no data available.

*Relapse ALL occurred after 11 months of clinical complete remission, but a Ph^1-positive bone marrow.

†Relapse ALL occurred after complete remission with Ph^1-negative bone marrow.

‡Common ALL immunophenotype but hematologic diagnosis of acute undifferentiated leukemia.

§CML occurred after 4 years of complete remission.

‖The karyotype of the ALL phase has been published.[39] During the 28 months of complete remission cytogenetic analysis of the bone marrow showed a normal karyotype with a residual standard t(9,22) in three out of 220 metaphases karyotyped. Leukemia relapsed as a Ph^1-positive AML that, after treatment, transformed in CML, chronic phase Fatal myeloid blast crisis occurred 4 months later

Southern blot analysis. DNA was prepared from bone marrow or peripheral blood samples as described.[24] DNA (10 μg) was digested with restriction enzymes, electrophoresed on 0.7% Agarose gels and blotted according to Southern.[25] Isolation of probes, hybridization, and washing conditions were as described in previous publications.[14,16] The *bcr* probes used are indicated in Fig 1.

In situ hybridization. Metaphases obtained from bone marrow or peripheral blood for cytogenetic analysis of the leukemic cells were also used for in situ hybridization. Technical procedures and c-*abl* probes used were as described.[26]

In one case (U3), where the banding was poor, grain distribution on the chromosomes not involved in the translocation was calculated for the chromosomes in morphological groups (A, B, C, etc). In cases of mosaicism (R5, R6) only the hybridization data on the Ph^1-positive metaphases have been tabulated. The expected number of grains based on the relative DNA content of the given chromosome according to Mendelsohn[27] was corrected for sex and translocation of part of chromosomal material.

RNA analysis. Total RNA was isolated using the LiCl/urea[28] method (K562, Hela, human testes, and bone marrow of ALL patient U6) or using the guanidine thiocyanate[20] method (bone marrow of patient R6 and peripheral blood of patient R2). Twenty μg of total RNA was electrophoresed on a 1% agarose gel in the presence of formaldehyde.[29] After blotting, the nitrocellulose filters were hybridized to c-*abl* and *bcr* probes as described.[22] Recombinant plasmid and cosmid clones were handled under the containment conditions following the guidelines of the Dutch Committee on Recombinant DNA Research.

RESULTS

bcr Rearrangements in ALL patients. DNA from the leukemic cells of 21 different ALL patients was screened on Southern blots using the *bcr* probes I to IV indicated in Fig 1. In the DNA of six ALL patients having leukemic cells without a Ph^1 chromosome or other karyotypic markers (Table 1), no rearrangements of the *bcr* could be detected. *Bgl*II-digested DNA, hybridized to *bcr* probes I and III, showed a hybridization signal with the normal 5.0-kb *Bgl*II fragment on chromosome 22 (L4, shown in Fig 2A). Similarly, *Hind*III-digested DNA showed the normal 10.0-, 1.8- and 4.5-kb fragments after hybridization with probes I, II, and III, respectively (data not shown). Additional hybridizing bands were not observed.

One case (R1) of Ph^1-positive ALL was studied after achievement of complete remission. Cytogenetic studies of bone marrow cells revealed a normal karyotype (46,XX). In the DNA made at this Ph^1-negative stage, only the normal *bcr* fragments were detected (Fig 2B). Of the 15 ALL patients showing a Ph^1 chromosome either in the acute or in the relapse phase, listed in Tables 2 and 3 the DNA of 14 patients was studied. Using different digests and probes we could detect *bcr* rearrangements in the DNA of nine of these patients. In five patients no *bcr* rearrangements were observed. In the DNA of eight Ph^1-positive ALL patients *bcr* rearrangements were observed. In three of these DNAs (U3, U4, and R6) only the newly generated fragment present on chromosome 22q− could be detected (R6, Fig 2E) besides the normal 5.0-kb *Bgl*II fragment. The rearranged 3′ *bcr* sequences (translocated to the 9q+ chromosome in CML) are not visible. Of patients R6 and U6 (Table 3) material was available from a relapse ALL phase. In each patient both *Bgl*II digested ALL- and relapse ALL-DNA showed identical aberrant *Bgl*II fragments (R6, Fig 2E).

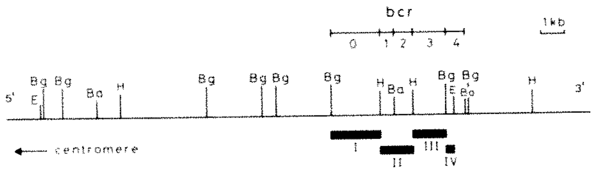

Fig 1. Restriction enzyme map of the human *bcr* locus on chromosome 22. *Bam*HI = Ba, *Bgl* II = Bg, *Eco*RI = E, *Hind* III = H. The 5.8-kb *bcr* indicated on top is artificially divided into five subregions (0-4). The probes used are indicated by solid boxes below the map. I, 2.0-kb Bg-H; II, 1.2-kb H-*Xho*II; III, 1.2-kb H-Bg; IV, 0.3-kb Bg-E.

Two of the ALL patients, L1 and R7 (Table 3) recurred with CML after 4 and 2 years of complete remission, respectively. Although the initial ALL phase of L1 was not karyotyped, the finding of a *bcr* rearrangement (Fig 2C) strongly suggests the presence of a Ph^1 chromosome. At the CML phase, a standard t(9;22) was found in the bone marrow cells, but unfortunately no DNA was available, and comparison of the molecular rearrangement of both phases was not possible. In patient R7, DNA from the ALL phase showed no *bcr* rearrangement; in contrast, DNA obtained in the CML phase did show a breakpoint in the *bcr* (Fig 2D). These results were confirmed by in situ hybridization of *bcr* probes showing segregation of the 5′ and 3′ *bcr* probes (data not shown).

Sublocalization of the bcr *breakpoints: Evidence for deletions in the* bcr. To localize the *bcr* breakpoints more precisely, we arbitrarily divided the 5.8-kb *bcr* into the five segments shown in Fig 1. Analogous to the mapping of the Ph^1 breakpoints in CML patients (16) we sublocalized the 5′ and/or 3′ breakpoints of the 10 *bcr* (+) DNAs (nine ALL and the CML phase of patient R7). Usually the break in chromosome 22 splits the *bcr* in two segments: the 5′ *bcr* segment, oriented towards the centromere and remaining on the 22q− chromosome, and a 3′ *bcr* segment that migrates to the 9q+ derivative of the standard Ph^1 translocation. These segments are identified by Southern blot analysis using the 5′ and the 3′ *bcr* probe (probes I and III, respectively, Fig 1). The results are given in Table 4. In six patients the breakpoint regions determined with various probes and digests are not located in the same subregion of the *bcr*. In two of these patients (L1 and L9) both breakpoints could be localized, although in a different subregion. In patient L1 probe I and probe III (5′ *bcr* and 3′ *bcr*, respectively, Fig 1) detect two different new hybridizing bands in *Bgl*II-digested L1 DNA. This indicates that both breakpoints are situated within the *bcr*. In *Hind*III-digested L1-DNA, probe II and probe III detect different extra hybridizing bands. Since probe II detects only the 5′ *bcr* segment in *Bgl*II-digested L1-DNA, the 5′ breakpoint is located in the 1.8-kb *Hind*III fragment (region 1 + 2). The 3′ breakpoint is located in segment 3 and a minimum of 300 bp of *bcr* sequences is deleted. (The distance between the 3′ end of probe II and the 5′ end of probe III.) In four other patients (U3, U4, R6, and R7) only one of the breakpoints could be assigned. For instance, in patient R6 the 5′ breakpoint could be mapped 3′ of the

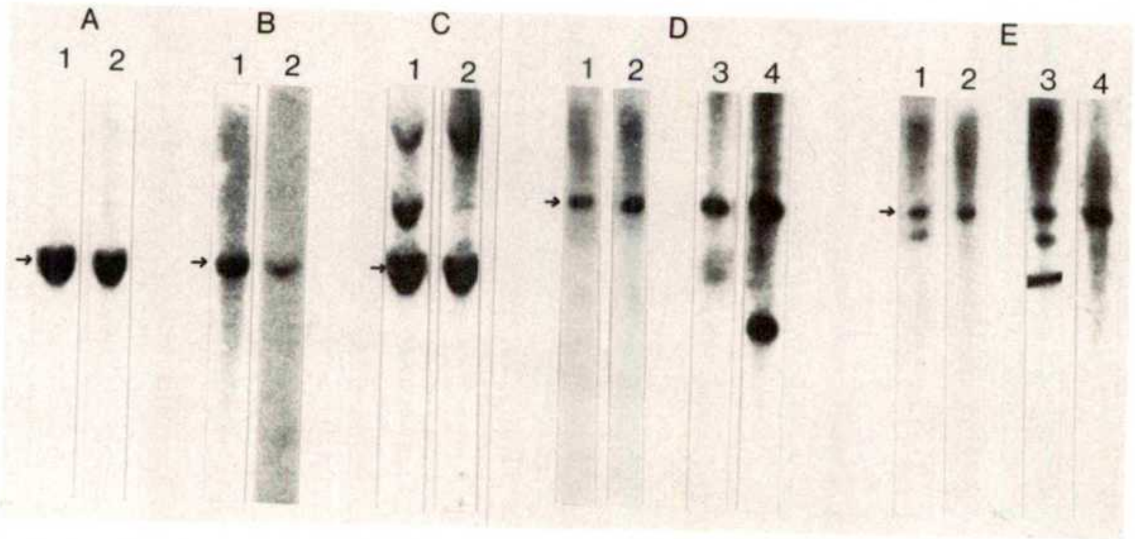

Fig 2. Southern blot analysis of *Bgl* II-digested DNA (10 μg) of the leukemic cells of five ALL patients. (A) ALL patient L4; (B) ALL patient R1; (C) ALL patient L1; (D) ALL (lane 1 and 2) and CML phase (lane 3 and 4) of patient R7; (E) ALL (lane 1 and 2) and relapse ALL (lane 3 and 4) of patient R6. Arrow indicates the 5.0 kb normal *Bql* II fragment. Lanes 1 and 3 are hybridized with probe I (5′ *bcr*) and lanes 2 and 4 are hybridized with probe III (3′ *bcr*) indicated in Fig 1. In (C), lane 1, the hybridization signal (top band) of a previous hybridization of the same filter with probe III (3′ *bcr*) is still visible.

*Bam*HI site in segment 2 of the *bcr*: abnormal *Bgl*II fragments with probe I and probe II, normal *Bam*HI fragments with probe I, and an aberrant *Hind*III fragment with probe II. Probes III or IV detect only the normal *bcr* fragments in either a *Bgl*II, *Hind*III, *Bam*HI, or *Eco*RI digest. Thus apparently the 3′ part of the *bcr* is deleted and the 3′ breakpoint of patient R6 maps outside the *bcr*.

In situ hybridization. Translocation of the c-*abl* oncogene was studied by in situ hybridization of c-*abl* specific sequences to metaphase chromosomes from five Ph^1-positive ALL patients (U3, U5, R2, R5, and R6). Distribution of silver grains was uniform and at random on all chromosomes except the specific signals ($P < 0.01$) on chromosomes 9(q34) and Ph^1 in all cases investigated (Table 5).

This indicates that c-*abl* sequences are translocated to the Ph^1 chromosome. In two of the patients (R2, R5) the *bcr* was not rearranged, which suggests that in these two cases the recombination of c-*abl* with chromosome 22 sequences is different from that in CML.

Table 4. Analysis of *bcr* Breakpoints

Patient No.	5′ Breakpoint in Segment	3′ Breakpoint in Segment
L8	1,2	1,2
L9	1,2	3*
U1	2	2
U2	1,2	1,2
U3	2	—*
U4	3	—*
R6†	2	—*
U6†	1	1
L1	1,2	3*
R7	—	2*

bcr segments are indicated in Fig 1.

*Deletion of *bcr* sequences has occurred.

†Identical breakpoints in initial and relapse phase.

c-abl *Transcription in ALL.* For the detection of c-*abl* mRNA a human 0.6-kb *Eco*RI-*Bam*HI DNA fragment was used as a probe. This fragment contains the most 5′ human v-*abl* hybridizing exon.[30] In order to examine whether the translocation resulted in a modification of the mRNA transcript as found in CML, RNA was isolated from the leukemic cells of 3 Ph^1-positive ALL patients. As a control, RNA isolated from human testes or Hela cells was used as well as the RNA of a Ph^1-positive CML blast crisis cell line, K562. As shown in Fig 3, in all RNA species the c-*abl*–specific probe detects the normal 6.0- and 7.0-kb mRNA transcripts. In addition a novel c-*abl* mRNA of 8.5 kb is present in the RNA of patients U6 (lane 2) and R6 (lane 4) as well as in K562 RNA (lane 3). This 8.5-kb mRNA is not present in the RNA of Hela cells (lane 1) or normal human testes (lane 6) or the leukemic cells of patient R2 (lane 5) exhibiting no *bcr* rearrangements in Southern blots.

The Northern blot containing the RNA of K562, patient R6, and patient R2, and human testes RNA was also hybridized to a 5′ *bcr* cDNA probe.[22] In all RNA species the normal *bcr* transcripts of 7.0 kb and 4.5 kb were present. In the RNA of patient R6 and K562 cells, this 5′ *bcr*-specific probe detects an additional hybridizing band of 8.5 kb (not shown).

DISCUSSION

Recently we showed that in CML the Ph^1 chromosome is the result of a consistent juxtaposition of the c-*abl* oncogene and the chromosome 22 *bcr* sequences.[16,26] In the present studies, using in situ hybridization, we demonstrate a translocation of the c-*abl* oncogene from chromosome 9 to chro-

Table 5. Results of In Situ Hybridization Studies With c-*abl* Probes

Patient No.	Cells Analyzed	Total of Grains	Chromosomes*	Grains on Chromosomes		
		On Chromosome/Background		Observed	Expected	χ^2
U3	26	189/68	5	5	6.0	0.2
			5q−	1	2.7	1.1
			9	13	4.5	16.1
			9q+	5	8.5	1.4
			22	3	1.6	1.2
			22q−	14	1.0	169.0
U5	43	243/75	9	27	5.8	77.5
			9q+	2	6.5	3.1
			22	0	2.1	2.1
			22q−	19	1.3	241.0
R2	30	113/54	9p−†	12	2.5	36.1
			9p+q+	4	3.1	0.3
			22	0	0.9	0.9
			22q−	5	0.6	32.3
R5	31	103/46	9p+‡	13	3.1	21.6
			9p+q+	3	3.7	0.1
			22	0	0.9	0.9
			22q−	9	0.5	144.5
R6	28	85/36	9	10	1.9	34.5
			9q+	2	2.1	0.0
			11	2	1.9	0.0
			11p+	2	1.9	0.0
			22	0	0.7	0.7
			22q−	7	0.4	108.9

*Karyotype of patients as given in Tables 2 and 3.

†9p− = der(9) t(3;9)(q27;p21); 9p+q+ = [4pter → p14::9p21 → q34::22q11 → qter]

‡9p+ = der(9) t(1;9)(q25;p21); 9p+q+ = [8qter → q13::9p21 → q34::22q11 → qter]

mosome 22 in five Ph^1-positive ALL patients. This observation proves that the Ph^1 chromosome in ALL is similar to that in CML[14] and derives from a reciprocal translocation between chromosome 9 and 22. The incidence of variant translocations (5/15) is higher than in CML, where only 5% to 8% of the patients show translocations different from t(9;22). However, in these variant translocations we also could demonstrate a translocation of the c-*abl* oncogene from 9q34 to the q11 band of chromosome 22.

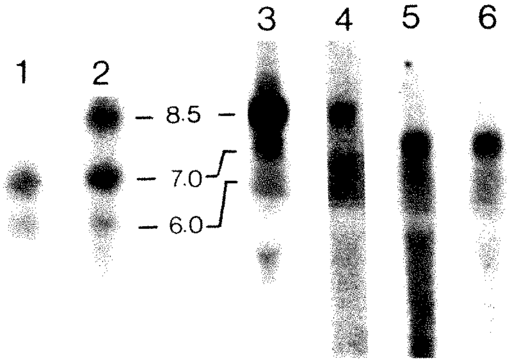

Fig 3. Northern blot analysis of RNA from three ALL patients and control cells. Total RNA (20 μg) was blotted and hybridized with a human c-*abl* probe. Lane 1, Hela cells; lane 2, ALL patient U6; lane 3, K562 cells; lane 4, ALL patient R6; lane 5, ALL patient R2; lane 6, human testes. The sizes (kb) of the hybridizing bands are indicated.

In CML, a breakpoint is consistently found in the 5.8-kb *bcr* of the Ph^1 chromosome.[16] In contrast, Southern blot analysis of *bcr* of the leukemic cells from 14 Ph^1-positive ALL patients only showed *bcr* rearrangements in nine patients. Sublocalization of the *bcr* breakpoints revealed that larger deletions involving *bcr* sequences frequently occurred in Ph^1-positive ALL patients as compared to Ph^1-positive CML patients. In the rare cases of CML where *bcr* deletions could be observed (D. Bootsma and A. de Klein, unpublished results), these measured 100 to 500 bp and did not result in a complete deletion of the 3′ *bcr* as in the ALL patients U3, U4, and R6. Although we did not observe *bcr* deletions during transition from chronic to acute phase of CML,[31] the 3′ *bcr* sequences are also absent in the cell line K562 established from pleural fluid of a CML patient in blast crisis.[22] However, cytogenetic analysis revealed a concordant absence of the 9q+ chromosome in K562,[32,33] whereas in Ph^1-positive ALL patients the derivative chromosome containing the deleted part of chromosome 22 was present. Studies of other Ph^1-positive hematologic disorders different from CML will be necessary to corroborate whether these large deletions are typical features of Ph^1-positive ALL.

Absence of *bcr* rearrangements were found in 5 cases of Ph^1-positive ALL. Recently, similar observations have been reported in two children with Ph^1-positive ALL.[34] However, in two of our cases studied by in situ hybridization it was clear that one copy of the c-*abl* had moved to chromosome 22 despite the lack of *bcr* rearrangement. Since the complete *bcr* gene has not yet been identified, the possibility remains that in these cases c-*abl* is translocated to the more unidentified 5′ sequences of the *bcr* gene. The demonstration of a breakpoint within the immunoglobulin light chain gene[35] (by in situ hybridization) in one Ph^1-positive ALL patient suggests that sequences which map more proximally on chromosome 22 than *bcr* can be involved in the Ph^1-translocation in ALL. These observations suggest that a proportion of Ph^1-positive ALL differs from CML by the absence of *bcr* rearrangements. The previously reported ALL cases were children, and the one childhood Ph^1-positive ALL case that we studied (R2) was also without detectable *bcr* rearrangement; but since four other cases with no *bcr* rearrangements were adults, this does not seem restricted to childhood Ph^1-positive ALL.

Ph^1-positive ALL patients have a worse prognosis as compared to the Ph^1-negative ALL patients.[36,37] In CML, Ph^1-negative patients with no translocation of c-*abl*[38] and no *bcr* rearrangements[16] seemed to belong to a distinct subclass of leukemia with a poorer prognosis.[4] Although the number of patients we studied is small, our data indicate no difference in survival time for the Ph^1-positive ALL patients with or without *bcr* rearrangements. A much larger series of ALL cases should be studied to ascertain any clinical relevance for diagnosis and prognosis of the distinction between Ph^1-positive ALL with or without *bcr* rearrangement.

Two Ph^1-positive ALL patients (U6 and R6) were studied at diagnosis and at relapse, with documented disappearance of the Ph^1 chromosome from the bone marrow during complete remission in one case (U6). Identical *bcr* rearrangements were observed in the initial and relapse ALL phase of each patient. This suggests that even if complete remission is achieved, the Ph^1-positive progenitor cell still can remain in the hematopoietic system.

As reviewed by others,[12,13] a rarely observed feature of Ph^1-positive ALL patients is that after achievement of complete remission, some recur with CML instead of ALL. Two such cases are included in this study: patients L1 and R7. Although we were unable, despite all efforts using frozen cells, to obtain a karyotype of patient L1, the finding of a *bcr* rearrangement strongly suggests that this was a Ph^1-positive ALL. The 4-year lapse between the ALL and CML phase makes it possible that CML is a secondary leukemia, but unfortunately we were not able to compare the *bcr* regions in these two phases, since no CML-DNA was available. Patient R7 showed a variant Ph^1 translocation in the initial ALL phase, and this variant Ph^1 was interpreted as a two-step rearrangement: first a standard t(9:22), followed by a t(9q+;14).[39] During the ALL phase this patient was without *bcr* rearrangements. When CML recurred a *bcr* breakpoint (with deletions) was found. This suggests either a secondary rearrangement of the original 9q+ or Ph^1 chromosome or the occurrence of a de novo Ph^1 translocation in another stem cell.

RNA isolated from Ph^1-positive CML or Ph^1-positive cell lines all contained the abnormally sized c-*abl* mRNA of approximately 8.5 kb.[19-22] In two of the three Ph^1-positive ALL patients analyzed a similar 8.5-kb transcript was present. Both these ALL patients contained *bcr* rearrangements, in contrast to the third patient, and in patient R6 the same mRNA hybridizes to a 5′ *bcr* probe. This suggests that, as in CML,[21-23] in ALL the juxtaposition of c-*abl* or *bcr* sequences can result in the transcription of a chimeric mRNA, which contains 5′ *bcr* and 3′ c-*abl* sequences. Translation of this chimeric mRNA would result in a *bcr*/*abl* fusion protein. Evidence for the presence of such a protein in Ph^1-positive CML cells and different CML cell lines has been reported.[40,41] An abnormally sized 210-kd c-*abl* protein is present in these cells which, in contrast to the normal 150-kd c-*abl*, has in vitro tyrosine kinase activity similar to the v-*abl* gene product.[42]

In the Ph^1-positive ALL cases studied, translocation of the c-*abl* oncogene to the 22q− chromosome has been demonstrated by in situ hybridization. However, regarding the observed heterogeneity in respect to the *bcr* rearrangements of Ph^1-positive ALL patients, the question remains open whether in all the ALL patients a *bcr*/c-*abl* chimeric mRNA is present. In those cases where neither *bcr* rearrangement nor an altered transcription of c-*abl* or *bcr* could be demonstrated, other genes or sequences could contribute to the generation and/or maintenance of ALL. The molecular aspects observed in some Ph^1-positive ALL patients are indistinguishable from CML, reinforcing the possibility that in some of these ALL patients there may be lymphoid blast crises developing from subclinical CML.[5,12] Investigation of more patients and follow-up for a longer time may therefore finally reveal the clinical and/or biologic importance of molecular differences among these patients.

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REFERENCES

1. Nowell PC, Hungerford DA: A minute chromosome in human chronic granulocytic leukemia. Science 132:1497, 1960

2. Rowley JD: A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290, 1973

3. Champlin RE, Golde DW: Chronic myelogenous leukemia: Recent advances. Blood 65:1039, 1985

4. Sandberg AA: Chromosomes in Human Cancer and Leukemia. New York, Elsevier/North Holland, 1979, p 262

5. Beard MEJ, Durrant J, Catovsky D, Wiltshaw E, Amess JL, Brearley RL, Kirk B, Wrigley PFM, Janossy G, Greaves MF, Galton DAG: Blast crisis of chronic myeloid leukaemia (CML). I. Presentation simulating acute lymphoid leukaemia (ALL). Br J Haematol 34:167, 1976

6. Oshimura A, Sandberg AA: Chromosomes and causation of human cancer and leukemia. XXV. Significance of the Ph^1 (including unusual translocations) in various acute leukemias. Cancer 40:1149, 1977

7. Abe S, Sanberg AA: Chromosomes and causation of human cancer and leukemia. XXXII. Unusual features of Ph^1-positive acute myeloblastic leukemia (AML), including a review of the literature. Cancer 43:2352, 1979

8. Bloomfield CD, Lindquist LL, Brunning RD, Yunis JJ, Coccia PF: The Philadelphia chromosome in acute leukemia. Virchows Arch [Cell Pathol] 29:81, 1978

9. Chessells JM, Janossy G, Lawler SD, Secker Walker LM: The Ph^1 chromosome in childhood leukemia. Br J Haematol 41:25, 1979

10. Priest JR, Robison LL, McKenna RW, Lindquist LL, Warkentin PI, LeBien TW, Wood WG, Kersey JH, Coccia PF, Nesbit ME: Philadelphia chromosome positive childhood acute lymphoblastic leukemia. Blood 56:15, 1980

11. LeBeau MM, Rowley JD: Recurring chromosomal abnormalities in leukaemia and lymphoma. Cancer Surveys 3:371, 1984

12. Catovsky D: Ph^1 positive acute leukaemia and chronic granulocytic leukemia: One or two diseases. Br J Haematol 42:493, 1979

13. Sandberg AA, Kohno S-I, Wake N, Minowada J: Chromosomes and causation of human cancer and leukaemia XLII Ph^1-positive ALL: An entity within myeloproliferative disorders? Cancer Genet Cytogenet 2:145, 1980

14. De Klein A, Geurts van Kessel A, Grosveld G, Bartram CR, Hagemeijer A, Bootsma D, Spurr NK, Heisterkamp N, Groffen J, Stephenson JR: A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. Nature 300:765, 1982

15. De Klein A, Hagemeijer A: Cytogenetic and molecular analysis of the Ph^1 translocation in chronic myeloid leukemia. Cancer Surveys 3:515, 1984

16. Groffen J, Stephenson JR, Heisterkamp N, De Klein A, Bartram CR, Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region, *bcr*, on chromosome 22. Cell 36:93, 1985

17. Heisterkamp N, Stam K, Groffen J, De Klein A, Grosveld G: Structural organization of the *bcr* gene and its role in the Ph^1 translocation. Nature 316:758, 1985

18. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, De Klein A, Bartram CR, Grosveld G: Localization of the c-*abl* oncogene adjacent to a translocation breakpoint in chronic myelocytic leukemia. Nature 306:239, 1983

19. Gale RP, Canaani E: An 8-kilobase *abl* RNA transcript in chronic myelogenous leukemia. Proc Natl Acad Sci USA 81:5648, 1984

20. Collins SJ, Kubonishi I, Miyoshi I, Groudine MT: Altered transcription of the c-*abl* oncogene in K562 and other chronic myelogenous leukemia cells. Science 225:72, 1984

21. Stam K, Heisterkamp N, Grosveld G, De Klein A, Verma RS, Coleman M, Dosik H, Groffen J: Evidence of a new chimeric *bcr*/c-*abl* mRNA in patients with chronic myelocytic leukemia and the Philadelphia chromosome. N Engl J Med 313:1425, 1985

22. Grosveld G, Verwoerd T, van Agthoven T, De Klein A, Ramachandran K, Heisterkamp N, Stam K, Groffen J: The chronic myelocytic cell line K562 contains a breakpoint in *bcr* and produces a chimeric *bcr*/c-*abl* transcript. Mol Cell Biol 6:607, 1986

23. Shtivelman E, Lifshitz B, Gale RB, Canaani E: Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukemia. Nature 315:550, 1985

24. Jeffreys AJ, Flavell RA: A physical map of the DNA regions flanking the rabbit β-globin gene. Cell 12:429, 1977

25. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

26. Bartram CR, Kleihauer E, De Klein A, Grosveld G, Teyssier JR, Heisterkamp N, Groffen J: c-*abl* and *bcr* are rearranged in a Ph^1-negative CML patient. EMBO J 4:683, 1985

27. Mendelsohn ML, Mayall BH, Bogart E, Moore DH, Perry BH: DNA content and DNA based centromeric index of the 24 human chromosomes. Science 179:1126, 1979

28. Auffray C, Rougeon F: Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. Eur J Biochem 107:303, 1980

29. Maniatis T, Fritsch EF, Sambrook J: Extraction, purification and analysis of mRNA from eukaryotic cells. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982, p 202

30. Wang JYJ, Ledley F, Goff S, Lee R, Groner Y, Baltimore D: The mouse c-*abl* locus: Molecular cloning and characterization. Cell 36:349, 1984

31. Bartram CR, De Klein A, Hagemeijer A, Carbonell F, Kleihauer E, Grosveld G: Additional c-*abl*/*bcr* rearrangement in a CML patient exhibiting a duplicated Ph^1-chromosome during blast crisis. Leuk Res 10:221, 1986

32. Selden JR, Emanuel BS, Wang E, Cannizzaro L, Palumbo A, Erikson J, Nowell PC, Rovera G, Croce CM: Amplified Cλ and c-*abl* genes are on the same marker chromosome in K562 leukemia cells. Proc Natl Acad Sci USA 80:7829, 1983

33. Chen TR: Modal karyotype of human leukemia cell line K562 (ATCC CCL 243). Cancer Genet Cytogenet 17:55, 1985

34. Rodenhuis S, Smets LA, Slater RM, Behrendt H, Veerman A: Distinguishing the Philadelphia chromosome of acute lymphoblastic leukemia from its counterpart in chronic myelogenous leukemia. N Engl J Med 313:51, 1985

35. Cannizzaro LA, Nowell PC, Belasco JB, Croce CM, Emanuel BS: The breakpoint in 22q11 in a case of Ph^1-positive acute lymphocytic leukemia interrupts the immunoglobulin light chain gene cluster. Cancer Genet Cytogenet 18:173, 1985

36. Bloomfield CD, Brunning RD, Smith KA, Nesbit ME: Prognostic significance of the Philadelphia chromosome in acute lymphocytic leukemia. Cancer Genet Cytogenet 1:229, 1980

37. Secker Walker LM: The prognostic implications of chromosomal findings in acute lymphoblastic leukemia. Cancer Genet Cytogenet 11:223, 1984

38. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, Heisterkamp N, Stephenson JR, Groffen J: Translocation of the human c-*abl* oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 306:277, 1983

39. Hagemeijer A, Bartram CR, Smit EME, van Agthoven AJ, Bootsma D: Is the chromosomal region 9q34 always involved in variants of the Ph^1 translocation? Cancer Genet Cytogenet 13:1, 1984

40. Konopka JB, Watanabe SM, Witte ON: An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell 37:1035, 1984

41. Konopka JB, Watanabe SM, Singer JW, Collins SJ, Witte ON: Cell lines and clinical isolates derived from Ph^1 positive chronic myelogenous leukemia patients express c-*abl* proteins with a common structural alteration. Proc Natl Acad Sci USA 82:1810, 1985

42. Davis CR, Konopka JB, Witte ON: Activation of the c-*abl* oncogene by viral transduction or chromosomal translocation generates altered c-*abl* proteins with similar in vitro kinase properties. Mol Cell Biol 5:204, 1985

Quantitative Relationship Between Heinz Body Formation and Red Blood Cell Deformability

By Walter H. Reinhart, Lanping A. Sung, and Shu Chien

The ultimate cause of destruction of red blood cells (RBCs) after oxidative damage with Heinz body formation is not well understood. We correlated the changes in RBC morphology and membrane protein composition after oxidant treatment with the alterations in deformability of whole cells and cell membranes. The incubation of RBCs with phenylhydrazine concentrations of 0.3 to 100 mg/dL at 37 °C for one hour led to a dose-dependent formation of Heinz bodies, ranging from isolated Heinz bodies at 1 mg/dL to a confluent coating of the inner membrane surface at 100 mg/dL phenylhydrazine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of a large quantity of hemoglobin bound to the ghost membrane of treated RBCs. Electrophoresis with and without dithiothreitol indicated that disulfide bridges are abundant between hemoglobin molecules and are also present among membrane proteins but are not the major bond between hemoglobin and membrane. Changes of spectrin, ankyrin, band 3, and band 6 and the appearance of a 260,000-dalton complex were also observed. With phenylhydrazine concentrations below 30 mg/dL, even in the presence of multiple Heinz bodies, the RBC deformability measured by filtration through 2.6-, 4.5-, and 6.8-µm pores and the membrane deformability determined by a filter aspiration technique were not altered. With 100 mg/dL phenylhydrazine, when the entire membrane was coated with Heinz bodies, RBC filterability and membrane deformability were drastically reduced. These results indicate that oxidative damage of RBCs with discrete Heinz body formation causes focal membrane rigidification but does not affect the global cellular deformability until the Heinz bodies nearly cover the entire cell endoface.

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AN INTRACELLULAR PRECIPITATION of hemoglobin with the formation of Heinz bodies occurs in congenital hemolytic anemias associated with unstable hemoglobins[1] and in certain congenital red blood cell (RBC) enzyme defects, eg, in glucose-6-phosphate dehydrogenase deficiency.[2] Heinz body formation can also be produced by oxidative agents, such as phenylhydrazine.[3] RBCs containing Heinz bodies are sequestered in the spleen, where the Heinz bodies are "pitted" or the entire cell is phagocytized by a macrophage.[4] Although this is well established, the ultimate cause of RBC destruction in oxidant-induced hemolytic disease is not well understood.

It is generally assumed that RBCs with Heinz bodies are less deformable.[5,6] RBC deformability is determined by the cell geometry, the viscoelastic properties of the cell membrane, and the internal fluid viscosity.[7] Oxidative damage with Heinz body formation can influence all these factors. These alterations in cell or membrane deformability could cause RBC sequestration in the spleen and facilitate erythrophagocytosis. It has been proposed that, due to the presence of Heinz bodies, the RBCs have a decreased filterability.[5,6,8] Other oxidative damages with possible influence on RBC deformability include the inhibition of the calcium-ATPase with concomitant intracellular accumulation of calcium,[9] the loss of potassium and water,[10] the peroxidation of membrane phospholipids with a subsequent polymerization of both membrane lipid and protein components,[11-14] and the change in the organization of the phospholipids within the membrane.[15]

In the present investigation we have correlated changes in cell morphology and membrane protein composition induced by the oxidant phenylhydrazine with the deformability of the RBCs and the cell membrane, with the aim of elucidating the role of deformability in the removal of oxidant-injured RBCs.

MATERIALS AND METHODS

Preparation of cells. Blood was drawn from healthy volunteers into heparinized Vacutainer tubes. The RBCs were washed three times in a Ringer solution containing 145 mmol/L NaCl, 4 mmol/L KCl, 2.2 mmol/L $CaCl_2$, 5.5 mmol/L dextrose, and 0.5 g/dL bovine albumin, adjusted to pH 7.4 with 0.1 N Tris buffer. The washed RBCs were resuspended in the Ringer solution to a hematocrit of 10.0%. Aliquots of this suspension were centrifuged at 1,500 g for two minutes to separate the RBCs from the suspending medium. A calculated volume of the suspending medium was then replaced by an equal volume of a stock solution of 500 mg/dL phenylhydrazine hydrochloride (molecular weight [mol wt] 144.6). The samples were resuspended and incubated aerobically in a waterbath at 37 °C, with gentle mixing from time to time.

Morphology of cells. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. The specimens were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.4, 4 °C) and postfixed in 2% OsO_4. For SEM, the specimens were dehydrated in ascending ethanol series, air-dried, coated with gold-palladium, and examined in a scanning electron microscope (JSM-25, Jeol Corp, Peabody, MA). For TEM, the specimens were embedded in Epon, and thin sections were prepared, stained with 2% uranyl acetate and lead citrate, and examined in a transmission electron microscope (Model EM 9, Carl Zeiss, Oberkochen, FRG).

The quantification of Heinz bodies was performed with standard morphometric techniques, as outlined by Weibel[16] and applied to the study of Heinz bodies by Weinstein et al.[5] The fractional volume of RBC cytoplasm occupied by Heinz bodies was obtained by point counting and expressed as volume percentage. The fraction of the

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cell membrane (expressed as percentage) in apposition with Heinz bodies was measured by intercept counting. The mean volume of a Heinz body was calculated using the method described by Weibel and Gomez[17]:

$$\overline{V}_{HB} = k\,\overline{S}^{3/2},$$

where k is a factor depending on the shape of the measured structure (k = 1.382 for spheres as assumed for Heinz bodies) and $\overline{S}$ is the mean cross-sectional area of a Heinz body calculated from diameter measurements on random sections in any direction.

In order to estimate the relative hemoglobin concentration in single RBCs with Heinz bodies, normal RBCs and RBCs treated with phenylhydrazine were fixed separately, mixed together, and then processed for TEM as described. A video digitizing system (Eye Com II, Model 109 PT, Spatial Data System, Goleta, CA) was used to perform densitometric scanning of microphotographs along a line that crossed both treated and untreated RBCs.

Analysis of membrane proteins. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis[18] was performed. RBCs washed in 0.01 mol/L phosphate buffer in 0.15 mol/L NaCl (pH 7.2) and resuspended in 0.02 mol/L phosphate buffer in 0.13 mol/L NaCl (with 5 mmol/L dextrose, 1 mmol/L adenine, and 10 mmol/L inosine, pH 7.4) were used. After treatment with phenylhydrazine (10 mg/dL for one hour or 100 mg/dL for two minutes), the RBCs were washed twice in ice-cold phosphate buffer, and RBC ghosts were prepared according to Bennett and Stenbuck.[19] The ghosts were washed four times. Membrane protein concentrations were determined by the method of Lowry et al.[20] A standard solution of hemoglobin monomer and oligomers was prepared by incubating a hemoglobin solution with 0.02% glutaraldehyde for 30 minutes at room temperature. SDS-polyacrylamide gel electrophoresis of the RBC ghosts and hemoglobin solution was performed according to Fairbanks et al[18] in the presence or absence of dithiothreitol (DTT). The gels were stained with Coomassie blue. The bands were numbered according to Steck et al.[21] Densitometric scanning was performed with the aid of the video digitizing system described.

Deformability of cells. For the determination of filterability, suspensions containing the same number of RBCs (hematocrit 10%) were incubated with phenylhydrazine at concentrations of 0.3, 1, 3, 10, 30, and 100 mg/dL, for one hour at 37 °C. The deformability was assessed by recording the filtration pressure with a pressure transducer (Model MP 45-14, Validyne Engineering Corp, Northridge, CA) during filtration of RBC suspensions under constant flow of 0.82 mL/min. The method has been described in detail elsewhere.[22] Polycarbonate filters (Nuclepore Corp, Pleasanton, CA) with measured pore diameters of 2.6 ± 0.2 μm, 4.5 ± 0.6 μm, and 6.9 ± 0.8 μm, respectively, were used. The relative resistance in a pore containing a RBC to that in a pore with Ringer solution alone, β, was computed as[23]:

$$\beta = 1 + [(P_i/P_o) - 1]V/h,$$

where P_i is the initial pressure reading, P_o is the pressure reading for the Ringer solution, V is the fraction of the pore volume occupied by RBC, and h is the fractional volume of RBCs in the suspension.

Membrane deformability. For the assessment of the deformability of RBC membrane, a modified version of a filter aspiration technique introduced by Brailsford et al[24] was used. A Nuclepore filter with a pore diameter of 1.0 μm was mounted horizontally in a filter chamber. The chamber was connected to a reservoir bottle filled with saline. The RBC suspension (hematocrit 10%) was placed onto the wetted filter. By adjusting the relative height of the reservoir bottle to the fluid level above the filter, a total hydrostatic pressure of 5 mm H_2O was imposed on the RBCs across the filter, leading to the aspiration of a small part of the RBC into the filter pores. After 20 seconds, 1% glutaraldehyde was added and the RBCs fixed for ten minutes while maintaining the constant hydrostatic pressure. The filters were then carefully removed and fixed in 1% glutaraldehyde overnight. The specimens were dehydrated in increasing ethanol series and air-dried. A SEM specimen stub covered with a piece of double-sided Scotch tape was placed onto the Nuclepore filter (with the side containing the cells facing up). The filter was then gently stripped away from the Scotch tape, leaving the RBCs on the Scotch tape with the pressure-aspirated segments (tongues previously in the pores) protruding vertically.[25] The specimens were coated and viewed in the scanning electron microscope at an angle of 80°. The length and the diameter of the protrusions were measured on the microscope screen at a magnification of 18,000×.

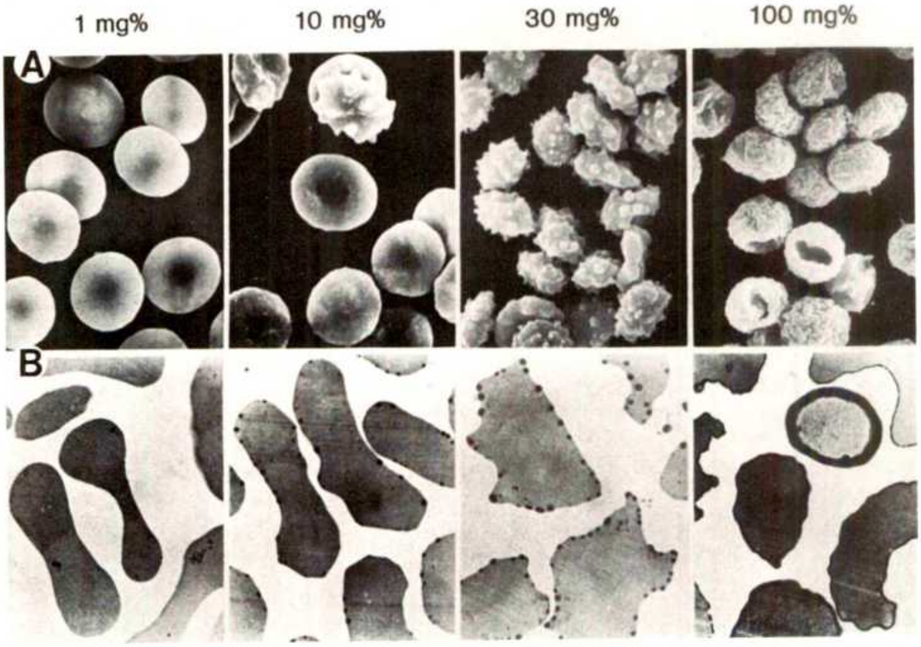

Fig 1. Scanning electron micrographs (A) and transmission electron micrographs (B) of red cells incubated for one hour at 37 °C with 1, 10, 30, or 100 mg/dL phenylhydrazine hydrochloride, as indicated.

Statistics. For statistical analysis, a one-way analysis of variance was used.

RESULTS

Morphology of cells. The incubation of RBCs with different concentrations of phenylhydrazine hydrochloride for one hour resulted in a dose-related formation of Heinz bodies in the cytoplasm (Fig 1). No Heinz bodies were detectable at a phenylhydrazine concentration of 0.3 mg/dL, 1 mg/dL induced Heinz bodies only occasionally, 10 and 30 mg/dL led to the formation of multiple Heinz bodies per cell, and 100 mg/dL generally resulted in a complete coating of the inner surface of the cell membrane with confluent Heinz bodies. The Heinz bodies were found to be in close apposition to the cell membrane. The portion of the cell surface containing the Heinz bodies showed an altered shape, ranging from a slight buckling to extended protrusions and severe membrane distortions, which gave the cell surface a bizarre appearance, like cerebral convolutions (Fig 1). At high magnifications (Fig 2) it can be seen that Heinz bodies were not confluent with the membrane, but that a small gap seemed to exist between the Heinz body and the membrane, which measured 10.7 ± 3.6 nm (mean ± SD of 25 measurements). The onset of Heinz body formation was rapid; two minutes after the beginning of the incubation, small Heinz bodies were already visible along the endoface of the membrane (not shown).

The morphometric data are given in Table 1. At phenylhydrazine concentrations >10 mg/dL, 100% of the cell sections showed Heinz bodies. The volume percentage of cytoplasm occupied by Heinz bodies gradually increased with increasing concentrations and reached 20% with 100 mg/dL phenylhydrazine. At this concentration, 97% of the cell membrane endoface had Heinz bodies. The volume of the individual Heinz body was similar for 3, 10, and 30 mg/dL.

The cytoplasmic hemoglobin concentration of RBCs exposed to phenylhydrazine (100 mg/dL for one hour) was compared with that of untreated RBCs (Fig 3). Densitometry of TEM micrographs revealed that Heinz bodies had an increased density; the relative density of the remainder of the cytoplasm was 0.52 ± 0.05 and 0.34 ± 0.08 ($P < .001$) for untreated (n = 43) and treated (n = 23) RBCs, respectively. Lower concentrations of phenylhydrazine (30 mg/dL and lower) had no significant influence on cytoplasmic density.

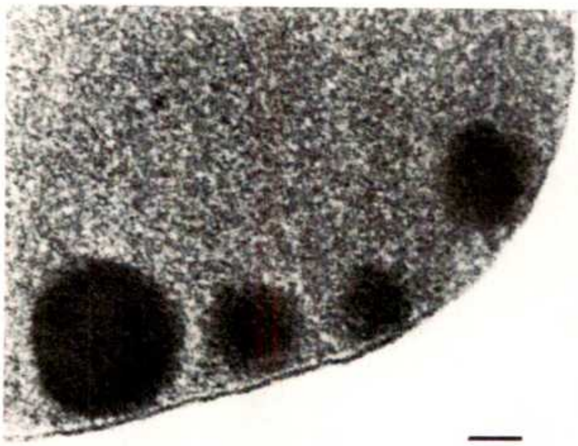

Fig 2. Transmission electron micrograph showing the attachment of Heinz bodies to the cell membrane. A small gap of about 10 nm between the Heinz bodies and the membrane can be seen. Bar = 100 nm.

Analysis of membrane proteins. The polyacrylamide gel electrophoresis on ghosts of RBCs treated with 10 mg/dL (one hour) and 100 mg/dL (two minutes) phenylhydrazine is shown in Fig 4; the densitometry of a gel from a similar experiment, in Fig 5. The incubation with 100 mg/dL phenylhydrazine was stopped after two minutes because it seemed to be more reasonable to study these early changes rather than the severe changes after one hour. Several alterations were found after phenylhydrazine treatment. (Hemo)globin was not found in controls, but was abundant in the ghosts of phenylhydrazine-treated cells, being visible by eye as a brown shade of the washed ghosts. The electrophoresis gel of the glutaraldehyde-treated hemoglobin solution (second well from right in Fig 4), which shows monomers, dimers (that band was split for an unknown reason), trimers, tetramers, and pentamers, is included as a reference. Electrophoresis of RBC ghosts with DTT showed a higher density of the hemoglobin monomer band (~14K) than that without DTT. After phenylhydrazine treatment (10 and 100 mg/dL) the gels also exhibited peaks corresponding to Hb dimer (between bands 7 and 8), which was weaker in the presence of DTT. In the absence of DTT, the 100 mg/dL phenylhydrazine specimen showed a peak corresponding to Hb trimer (between bands 5 and 6) as well (Fig 5B). While the control gels showed the typical pattern of major membrane proteins, ghosts from RBCs treated with 100 mg/dL phenylhydrazine had some smearing between the protein bands on the gel. The smearing was more pronounced in the absence of DTT, which also led to reduced amounts of bands 1, 2, and 2.1. These findings indicate that S-S bonds may be involved, at least in part, in forming molecular complexes containing these bands. The higher density of band 6 after 10 mg/dL phenylhydrazine suggests that a newly formed complex comigrated with band 6. With 100 mg/dL phenylhydrazine, band 6 disappeared, suggesting that the comigrating complex and band 6 itself were bound to other complexes or that the high dose of phenylhydrazine dissociated the loosely bound band 6 from the membrane.[26] For both phenylhydrazine concentrations, a sharp band appeared at the lower boundary of band 3. Above band 1 a faint band of high mol wt (approximately 260,000 daltons) was detected (Fig 4).

Deformability of cells. The results of the RBC filtration experiments are shown in Fig 6. Up to a phenylhydrazine concentration of 10 mg/dL, the relative resistance of a RBC in a pore (β) was not different from control values for all three pore sizes. A concentration of 30 mg/dL resulted in an increase of β in 2.6-μm pores (threefold), but not in 4.5- and 6.9-μm pores. The concentration of 100 mg/dL had a dramatic effect on RBC filtration for all three pore sizes. The RBCs virtually could not pass 2.6- and 4.5-μm pores, and β for 6.9-μm pores was increased 20-fold.

Counting of the number of Heinz bodies per cell section on TEM pictures showed that it was unaffected by filtration

Table 1. Morphometric Data of Heinz Bodies (HB)*

	Phenylhydrazine Concentration (mg/dL)			
	3	10	30	100
Cell sections with HB (%)	22	97	100	100
Cytoplasm occupied by HB (%)	0.2 ± 0.2	1.8 ± 0.7	5.0 ± 1.4	19.8 ± 4.1
Membrane area adjacent to HB (%)	0.3 ± 0.3	7.8 ± 2.4	18.8 ± 3.2	97.3 ± 1.7
Mean cross-sectional diameter of HB (µm)	0.17 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	Confluence
Mean volume of single HB (10^{-3} µm^3)	4.86	5.37	4.65	Confluence

*Values are mean ± SEM. The data for the first three lines were obtained from 12 to 18 cells, and the results for the last two lines were obtained from 50 Heinz bodies.

through 2.6-µm pore filters. Thus, for 10 mg/dL phenylhydrazine, the number was 4.2 ± 2.1 (mean ± SD) per cell section before and 4.2 ± 2.2 after filtration; for 30 mg/dl phenylhydrazine, 18.3 ± 5.9 before and 21.5 ± 7.8 after filtration.

Membrane deformability. The results of the membrane deformability study are shown in Fig 7. The length-radius ratio of the aspirated protrusions was used as a parameter for estimating membrane deformability. Phenylhydrazine concentrations up to 10 mg/dL did not affect the membrane deformability (Fig 7A through C); the length-radius ratio was 2.56 ± 0.38, 2.65 ± 0.35, and 2.74 ± 0.34 (mean ± SD, n = 40) for 0, 3, and 10 mg/dL phenylhydrazine, respectively. With 30 mg/dL phenylhydrazine (Fig 7D), the length-radius ratio was significantly reduced (1.89 ± 0.30, $P < .001$); with 100 mg/dL (Fig 7F), there was no detectable deformation (the small nodules were due to Heinz body attachment, as shown in Fig 1). The transmission electron micrograph for 30 mg/dL phenylhydrazine (Fig 7E) shows that the large number of Heinz bodies present may leave insufficient amount of intervening membrane area for deformational entry into the pore, and thus reduce the protrusion length.

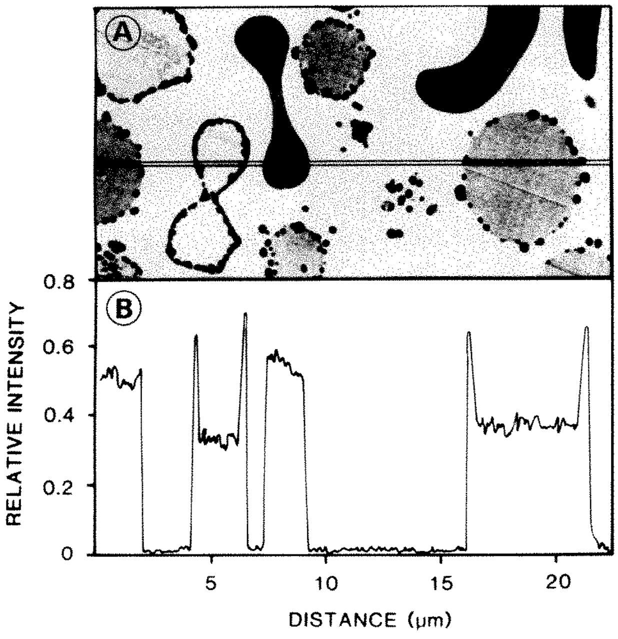

Fig 3. Estimation of the relative hemoglobin concentration in RBCs containing Heinz bodies. (A) Untreated and treated (100 mg/dL phenylhydrazine for one hour) cells were fixed separately and then mixed and embedded together. Densitometric scanning was performed on the transmission electron micrographs along a line that crossed both treated and untreated cells. (B) Tracing of the densitometry performed along a line indicated in the top graph. The relative density of the cytoplasm was measured: Heinz bodies have a higher density and the cytoplasm of these cells a lower density than do controls.

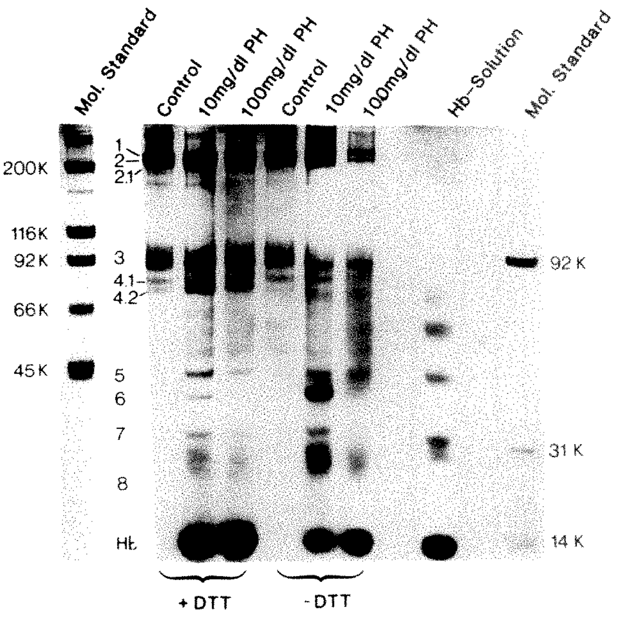

Fig 4. Polyacrylamide gel electrophoresis of ghost membrane proteins in the presence (+DTT) or absence (−DTT) of dithiotreitol, an agent that reduces S-S bonds. The RBCs were treated with 10 mg/dL phenylhydrazine for one hour or 100 mg/dL phenylhydrazine for two minutes at 37 °C. A known volume (35 µL) of ghost membrane was dissolved in the solubilization solution (65 µL), and 40 µL of each solution was applied to each lane. Each lane contained 50 µg of ghost membrane protein. Protein bands are numbered according to Steck.[21] High- (left margin) and low- (right margin) mol-wt standards are shown. A hemoglobin solution treated with 0.02% glutaraldehyde for 30 minutes was electrophoresed with DTT simultaneously, showing (from top to bottom) penta-, tetra-, tri-, di-, and mono-mers of (hemo)globin. The gel was stained with Coomassie blue.

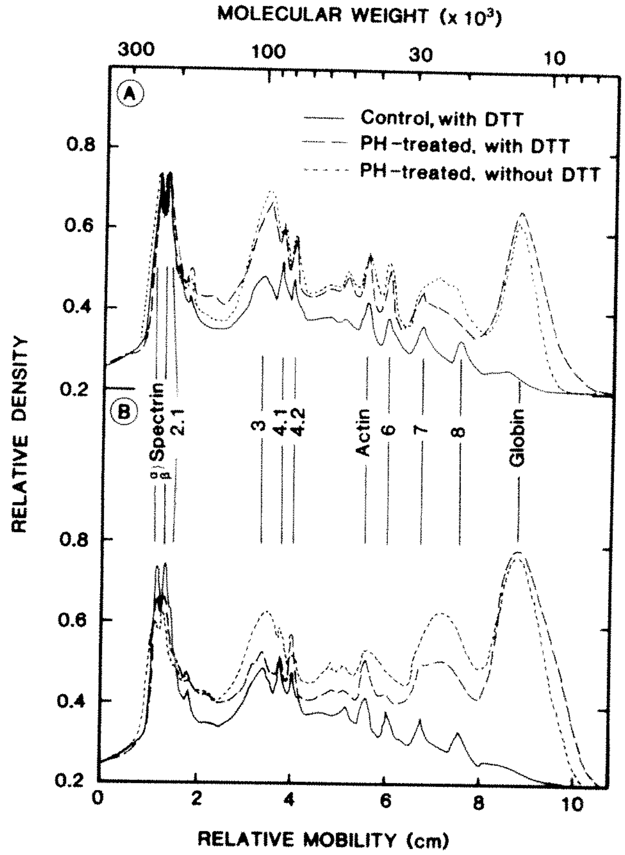

Fig 5. Densitometry of a polyacrylamide gel after membrane protein electrophoresis of untreated and phenylhydrazine-treated RBCs in the presence or absence of DTT. (A) Treated with 10 mg/dL phenylhydrazine for one hour at 37 °C. (B) Treated with 100 mg/dL phenylhydrazine for two minutes at 37 °C.

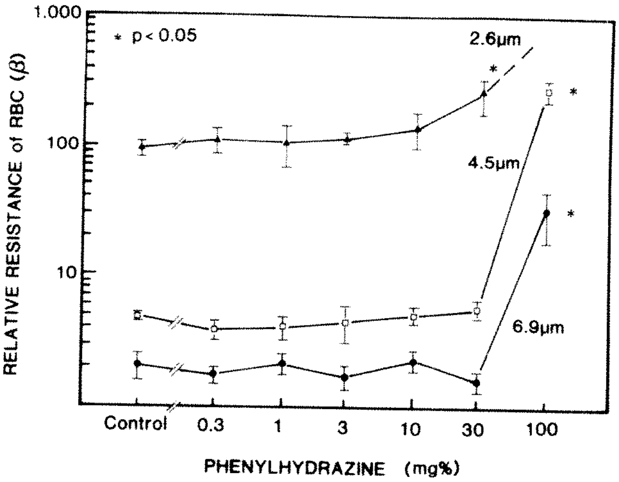

Fig 6. Log-log plot of the relative resistance for a red cell in a filter pore (β) against the concentration of phenylhydrazine for three pore sizes (2.6-, 4.5-, and 6.9-µm). The data are means ± SD of four experiments. Asterisks indicate $P < .05$.

DISCUSSION

The formation of Heinz bodies in intact RBCs induced by phenylhydrazine is dose- and time-dependent. Concentrations below 1 mg/dL did not induce any Heinz bodies, indicating that fresh RBCs with normal glutathione levels are capable of compensating for the oxidative effect of such phenylhydrazine concentrations for at least one hour. The other extreme was the complete coating of the cellular membrane with a continuous layer of Heinz bodies observed at a concentration of 100 mg/dL after one hour. The oxidative injury may vary with the redox agent used, and it would be interesting to obtain such dose-response curves for other oxidants.

With few exceptions, all Heinz bodies were found in proximity to the cell membrane. It has been claimed that Heinz bodies form in the cytoplasm and migrate to the membrane with time.[27] Our findings suggest that the formation takes place in close relation with the membrane. This may be the result of the vulnerability of the outermost layer of hemoglobin when the oxidative agent diffuses into the red cell or an early involvement of the membrane constituents, eg, phospholipids and membrane skeleton proteins.

We found a thin space of 10 nm between the Heinz bodies and the membrane on TEM at high magnification (Fig 2). Although we cannot exclude a fixation artifact, it could indicate that the denatured hemoglobin binds to components of the membrane skeleton that underlie the lipid bilayer, eg, spectrin. This hypothesis is supported by the observation of Ziparo et al,[28] who have shown that ferritin-conjugated antispectrin antibodies line up against the membrane, with the iron core of the ferritin approximately 10 nm apart from the apparent edge of the cell membrane.

How Heinz bodies are bound to membrane constituents has been a subject of debate for some time. Earlier investigations suggested that disulfide bonding may account for the attachment of unstable hemoglobin to the membrane[29]; others have found that the membrane attachment of Heinz bodies was through hydrophobic bonding.[30,31] Our results from polyacrylamide gel electrophoresis (Figs 4 and 5) showed that without DTT, ie, with intact S-S bridges, hemoglobin oligomers appeared in the phenylhydrazine-treated specimens at the expense of hemoglobin monomer, which supports the contention that S-S bridges are formed between (hemo)globin chains during oxidative hemoglobin denaturation (Heinz body formation) and are responsible for most oligomer formation.[32,33] If Heinz bodies were associated with spectrin by S-S bonds, one would expect to see a high-mol-wt complex above spectrin on the gel in the absence of DTT. Electrophoresis of ghost membrane treated with 100 mg/dL phenylhydrazine without DTT, however, revealed little, if any, high-mol-wt complex larger than spectrin. This suggests that S-S bridges are probably not the major type of bonding between the denatured hemoglobin and spectrin. The smearing distribution of proteins between bands 3 and 5 may be the result of an association between the denatured hemoglobin with membrane proteins other than spectrin or an association among various membrane proteins. The prevention of this smearing with the addition of DTT indicates the involvement of S-S bonds in these associations.

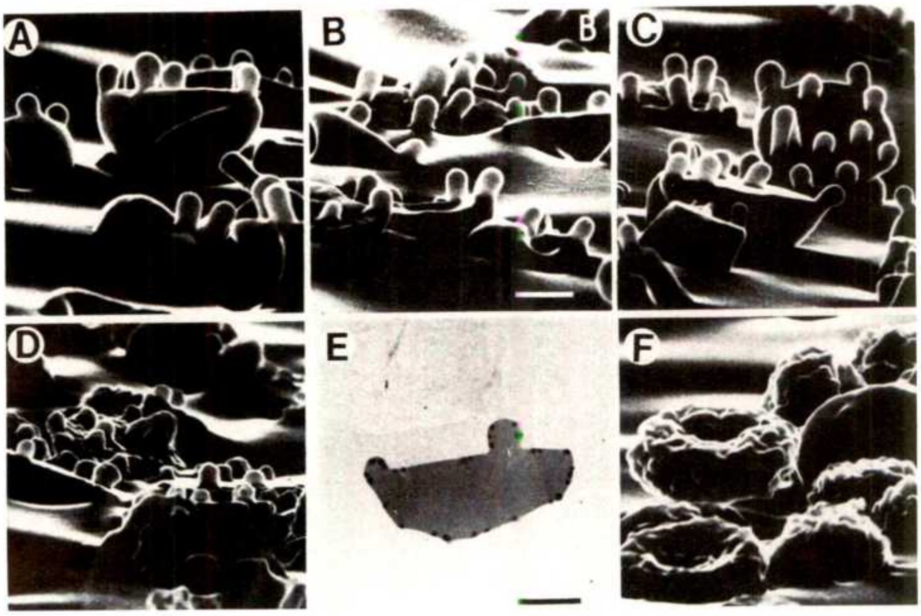

Fig 7. Assessment of the membrane deformability of RBCs with a filter aspiration technique.[24,25] The scanning electron micrographs show the protrusions produced by a hydrostatic pressure of 5 mm H_2O for RBCs treated with (A) 0 mg/dL, (B) 3 mg/dL, (C) 10 mg/dL, (D) 30 mg/dL, and (F) 100 mg/dL phenylhydrazine. The length-radius ratio of the protrusions was, for (A), 2.56 ± 0.38 (mean ± SD, n = 40); for (B), 2.65 ± 0.35; for (C) 2.74 ± 0.34; for (D) 1.89 ± 0.30 ($P < .001$); and for (F) zero. The transmission electron micrograph shown for 30 mg/dL phenylhydrazine (E) demonstrates that at this concentration (and above), the decrease in membrane area unoccupied by the large number of Heinz bodies can cause a reduced penetration length into the pore. The bars represent 2 μm.

Of special interest are the minor complex with a mol wt of about 260,000 daltons, the sharp band within band 3, and the disappearance of band 6 with high phenylhydrazine concentrations. Palek et al[32] found a 260,000-dalton complex in RBCs deficient in glucose-6-phosphate dehydrogenase that were treated with an oxidant (acetylphenylhydrazine). A 255,000-dalton complex formed in vivo in senescent RBCs has recently been described by Snyder et al,[34] who assumed a spectrin-hemoglobin crosslinking due to an oxidative damage occurring in vivo. It has been shown that denatured hemoglobin binds with high affinity to the cytoplasmic domain of band 3, where it competes for the binding with band 6,[35] and that band 3 becomes clustered in phenylhydrazine-treated RBCs.[36] Changes in the transmembraneous protein band 3 could play an important role in the recognition of damaged areas of RBCs by macrophages.

The present filtration study demonstrates that the presence of a limited number of small Heinz bodies in the RBC does not affect cell deformability. Apparently, the cytoplasmic viscosity and membrane viscoelasticity, two of the determinants of cell deformability,[7] are not influenced by small quantities of denatured hemoglobin underlying the membrane endoface. The cytoplasmic viscosity is determined by the remainder of the hemoglobin in the cell interior; the cytoplasmic hemoglobin concentration is near the control level for 30 mg/dL phenylhydrazine, and actually lower for an incubation of one hour with 100 mg/dL, where no loss of cell water has yet occurred.[10] When Heinz bodies are attached to the inner surface of the cell membrane, this area of the membrane becomes rigid, as can be seen by the small bumps of the membrane. Beside these rigid areas that overlay the Heinz bodies, the membrane is presumably not altered in its flexibility, analogous to the canvas parts of a tent. Thus, at a phenylhydrazine concentration of 30 mg/dL, 20% of the membrane becomes rigidified, while the other 80% is essentially normal (Table 1). Such a cell is still capable of passing through narrow channels, eg, the 4.5- and 6.9-μm pores; its passage through 2.6-μm pores, however, is already impaired. A higher concentration of phenylhydrazine (100 mg/dL) results in the involvement of the entire cell membrane, which is now plastered with denatured hemoglobin (Fig 1). These cells are unable to traverse 2.6- and 4.5-μm pores and barely pass through 6.9-μm pores.

Three other filtration studies on RBCs containing Heinz bodies have been reported. In these studies RBCs were incubated at a single high concentration of monomethylhydrazine,[5] acetylphenylhydrazine,[6] and phenylhydrazine[8] over a period of several hours, and RBC filterability was tested through 5-μm pores[5,6] and 8-μm pores[8] under a constant, high negative pressure. RBC filterability decreased progressively between two and five hours of incubation, but there was no significant change after a one-hour incubation. This latter finding is in agreement with our results with 4.5- and 6.9-μm pores for phenylhydrazine concentrations ≤30 mg/dL. Weinstein et al[5] performed a morphometric quantification of the Heinz bodies induced by 0.02 mol/L monomethylhydrazine. After six-hour incubation they found 5.2% of the cell membrane in contact with Heinz bodies and 5.2% of the cytoplasm occupied by Heinz bodies. These values are comparable to our results on RBCs incubated in 10 to 30 mg/dL phenylhydrazine for one hour. In contrast to our findings on RBC filtration, this small amount of Heinz bodies decreased the RBC filterability in the study of Weinstein et al[5] by a factor of >50. One possible explanation for the discrepancy between the two studies is that the six-hour incubation with a different oxidative compound damaged the remainder of the cell (membrane or cytoplasm) more profoundly than the number of Heinz bodies per se, eg, by the loss of potassium and cell water,[10] or a slow disulfide exchange between the denatured hemoglobin and spectrin.[8] A four-hour incubation of RBCs with acetylphenylhydrazine also resulted in decreased deformability as measured by ektacytometry.[37] Another possible explanation is the difference in filtration methodology. We used a constant flow and measured the initial rise of filtration pressure, whereas the previous authors used a constant pressure and measured the filtration time for a volume of 4 to 20 mL. Plugging of filter pores by cells is progressive with time[38]; therefore, their results obtained from the filtration of such a volume probably reflect more the plugging effect of cells containing Heinz bodies. In our experiments, however, Heinz bodies up to 5% of the cytoplasm did not result in a higher rate of pore plugging than control, as calculated from the rate of pressure rise with time[38] over the period of measurement (not shown).

Cell filtration is commonly used to assess RBC deformability in a variety of disease states and its alterations induced by drug actions. Our results show that focal changes of the membrane or cytoplasm do not affect the global RBC filterability. This finding has significant implications in the interpretation of filterability tests in clinical investigations; thus, a normal filterability, especially through larger pores, does not rule out such focal abnormalities in RBCs.

Moderate degrees of oxidative damage and Heinz body formation with phenylhydrazine up to 10 mg/dL did not affect the membrane deformability as assessed by the filter aspiration method using 1-μm pores. This indicates that the RBC membrane between Heinz bodies has normal elastic properties, although the portion of the membrane associated with Heinz bodies has become rigid. An increase in phenylhydrazine concentration to 30 mg/dL caused a significant decrease in membrane deformability. This may result from a reduced deformability of the RBC membrane between Heinz bodies or the insufficient areas of such residual membrane between large numbers of Heinz bodies to effect a full deformational entry into the pore. The complete coating of the membrane endoface with precipitated hemoglobin seen after 100 mg/dL phenylhydrazine rigidified the entire membrane such that no membrane deformation occurred in response to the applied aspiration pressure of 5 mm H_2O. The results with a moderate Heinz body formation (<30 mg/dL phenylhydrazine), which are probably more relevant to pathophysiological conditions in vivo, indicate that the deformability of the RBC membrane between Heinz bodies remains unaffected.

Our results suggest that a limited number of small Heinz bodies per se do not affect cell deformability. It is therefore unlikely that in such diseases as glucose-6-phosphate dehydrogenase deficiency[2,39] or unstable hemoglobins,[1,33] the Heinz bodies cause the change in RBC deformability and the in vivo hemolysis. Red cells containing giant Heinz bodies, which are larger than the opening of the slit in the splenic sinus wall to be passed, will naturally be trapped and undergo erythrophagocytosis.[4] This, however, is probably an uncommon situation in vivo. Red cells with smaller Heinz bodies are most likely not sequestered by their "filterability" characteristics in the spleen. It seems more likely that macrophages recognize the membrane areas with attached Heinz bodies by their focal rigidity or biochemical changes of the membrane, eg, changes in the integral membrane proteins band 3 and glycophorins. Depending on the size of the involvement, this would result in either a focal pitting or an extended engulfment with subsequent destruction of the whole RBC. Thus, our studies suggest that an oxidative damage with a limited number of small Heinz bodies represents primarily a biochemical hazard for the RBC (eg, a loss of water and potassium[10]) and that a significant coverage of membrane endoface by Heinz bodies is needed for the RBC to encounter biophysical disturbances in the microcirculation.

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REFERENCES

1. Carrell RW, Lemann H: The unstable hemoglobin hemolytic anemias. Semin Hematol 6:116, 1969

2. Beutler E: Glucose-6-phosphate dehydrogenase deficiency, in Williams WJ, Beutler E, Erslev AJ, et al (eds): Hematology. New York, McGraw-Hill, 1977, p 466

3. Jandl JH, Engle LK, Allen DW: Oxidative hemolysis and precipitation of hemoglobin. I. Heinz body anemias as an acceleration of red cell aging. J Clin Invest 39:1818, 1960

4. Rifkind RA: Heinz body anemia: An ultrastructural study. II. Red cell sequestration and destruction. Blood 26:433, 1965

5. Weinstein RS, George ME, Steingart RH: Contribution of Heinz bodies to alterations in red cell deformability. Toxicol Appl Pharmacol 32:545, 1975

6. Lubin A, Desforges JF: Effect of Heinz bodies on red cell deformability. Blood 39:658, 1972

7. Chien S: Biophysical behavior of red cells in suspension, in MacN Surgenor D (ed): The Red Blood Cell. Orlando, FL, Academic, 1975, p 1031

8. Vilsen B, Nielsen H: Reaction of phenylhydrazine with erythrocytes. Cross-linking of spectrin by disulfide exchange with oxidized hemoglobin. Biochem Pharmacol 33:2739, 1984

9. Shalev O, Leida MN, Hebbel RP, et al: Abnormal erythrocyte calcium homeostasis in oxidant-induced hemolytic disease. Blood 58:1232, 1981

10. Orringer EP, Parker JC: Selective increase of potassium permeability in red blood cells exposed to acetylphenylhydrazine. Blood 50:1013, 1977

11. Jain SK, Hochstein P: Generation of superoxide radicals by hydrazine: Its role in phenylhydrazine induced hemolytic anemia. Biochim Biophys Acta 586:128, 1979

12. Jain SK, Hochstein P: Polymerization of membrane components in aging red blood cells. Biochem Biophys Res Commun 92:247, 1980

13. Rice-Evans C, Hochstein P: Alterations in erythrocyte membrane fluidity by phenylhydrazine-induced peroxidation of lipids. Biochem Biophys Res Commun 100:1537, 1981

14. Johnson GJ, Allen DW, Flynn TP, et al: Decreased survival in vivo of diamide-incubated dog erythrocytes. J Clin Invest 66:955, 1980

15. Jain SK: In vivo externalization of phosphatidylserine and phosphatidylethanolamine in the membrane bilayer and hypercoagulability by the lipid peroxidation of erythrocytes in rats. J Clin Invest 76:281, 1985

16. Weibel ER: Stereological principles for morphometry in electron microscopic cytology. Int Rev Cytol 26:235, 1969

17. Weibel ER, Gomez DM: A principle for counting tissue structures on random sections. J Appl Physiol 17:343, 1962

18. Fairbanks G, Steck TL, Wallach DFH: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606, 1971

19. Bennett V, Stenbuck PJ: Human erythrocyte ankyrin. Purification and properties. J Biol Chem 255:2540, 1980

20. Lowry OH, Rosenbrough NJ, Farr AL, et al: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951

21. Steck TL: Cross-linking the major proteins of the isolated erythrocyte membrane. J Mol Biol 66:295, 1972

22. Reinhart WH, Usami S, Schmalzer EA, et al: Evaluation of red blood cell filterability test: Influences of pore size, hematocrit level and flow rate. J Lab Clin Med 104:501, 1984

23. Reinhart WH, Chien S: Roles of cell geometry and cellular viscosity in red cell passage through narrow pores. Am J Physiol 248:C473, 1985

24. Brailsford JD, Korpman RA, Bull BS: The aspiration of red cell membranes into small holes: New data. Blood Cells 3:25, 1977

25. Baker RF: Membrane deformability of metabolically depleted human red cells. Blood Cells 7:551, 1981

26. Shin BC, Carraway KL: Association of glyceraldehyde 3-phosphate dehydrogenase with the human erythrocyte membrane. Effect of detergents, trypsin, and adenosine triphosphate. J Biol Chem 248:1436, 1973

27. Rifkind RA, Danon D: Heinz body anemia—An ultrastructural study. I. Heinz body formation. Blood 25:885, 1965

28. Ziparo E, Lemay A, Marchesi VT: The distribution of spectrin along the membranes of normal and echinocytic human erythrocytes. J Cell Sci 34:91, 1978

29. Jacob HS, Brain MC, Dacie JV: Altered sulfhydryl reactivity of hemoglobins and red blood cell membranes in congenital Heinz body hemolytic anemia. J Clin Invest 47:2664, 1968

30. Winterbourn CC, Carrell RW: The attachment of Heinz bodies to the red cell membrane. Br J Haematol 25:585, 1973

31. Chan E, Desforges JF: The role of disulphide bonds in Heinz body attachment to membranes. Br J Haematol 33:371, 1976

32. Palek J, Liu SC, Snyder LM: Metabolic dependence of protein arrangement in human erythrocyte membranes. I. Analysis of spectrin-rich complexes in ATP-depleted red cells. Blood 51:385, 1978

33. Winterbourn CC, Carrell RW: Studies of hemoglobin denaturation and Heinz body formation in the unstable hemoglobins. J Clin Invest 54:678, 1974

34. Snyder LM, Leb L, Piotrowski J, et al: Irreversible spectrin-haemoglobin crosslinking in vivo: A marker for red cell senescence. Br J Haematol 53:379, 1983

35. Waugh SM, Low PS: Hemichrome binding to band 3: Nucleation of Heinz bodies on the erythrocyte membrane. Biochemistry 24:34, 1985

36. Low PS, Waugh SM, Zinke K, et al: The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. Science 227:531, 1985

37. Morimoto M, Feo CJ: Effet des corps de Heinz produits in vitro sur la deformabilité des erythrocytes. Mesure par une technique diffractometrique (ektacytometre). Resultats preliminaires. Nouv Rev Fr Hematol 22:53, 1980

38. Chien S, Schmalzer EA, Lee MML, et al: Role of white blood cells in filtration of blood cell suspensions. Biorheology 20:11, 1983

39. Johnson GJ, Allen DW, Cadman S, et al: Red-cell-membrane polypeptide aggregates in glucose-6-phosphate dehydrogenase mutants with chronic hemolytic disease. A clue to the mechanism of hemolysis. N Engl J Med 301:522, 1979

Only Adult Hemoglobin Is Produced in Fetal Nonerythroid x MEL Cell Hybrids

By S. Takegawa, M. Brice, G. Stamatoyannopoulos, and T. Papayannopoulou

In order to test whether the ontogenetic origin of human parental cells influences the expression of globin genes, we did fusions of mouse erythroleukemia (MEL) cells with fetal or adult nonerythroid cells and we assessed globin expression in hybrids. For selection of hybrid clones we used a monoclonal antibody that recognizes a human chromosome 11-linked cell surface marker. Globin expression was assessed with fluorescent antibody labeling, globin isoelectric focusing, and S1 nuclease mapping. Chromosome 11-containing hybrids from human nonerythroid cells (adult or fetal lymphoid cells; fetal fibroblasts) produced human adult (β) but not fetal (γ) globin chains. These results suggest that the MEL x nonerythroid cell hybrids express the adult human globin independent of whether the human cells derive from the fetal or from the adult stage of human development. Our findings, together with previous observations in MEL x fetal erythroid or MEL x HEL hybrids, show that the globin program of the parental human cell is the main determinant of the expression of fetal human globin in these hybrids.

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SOMATIC CELL HYBRIDIZATION, more specifically, the fusion of mouse erythroleukemia (MEL) cells with various types of human cells, has been used as a tool in the analysis of human globin switching.[1-4] Previous studies have shown that transfer of human chromosomes from adult bone marrow cells,[1] lymphoblasts,[2] or fibroblasts[3] into MEL cells leads to inducible expression of human globin genes in the hybrids. However, only β- and not γ-globin expression is activated after these chromosomal transfers.[2,3] In contrast, we have observed expression of mainly γ-globin in hybrids of MEL cells with human fetal erythroid cells.[4] The latter observations have been interpreted as evidence that the program of globin in the parental cell determines whether there will be γ expression in the hybrids. An alternative possibility is that the fetal human x MEL cell hybrids express γ-globin because of the developmental derivation of the human parental cell from the fetal stage of human ontogeny.

To test whether the origin of the human cells from the fetal stage of ontogeny influences the expression of the fetal or adult globin genes in the hybrids, we fused MEL cells with fetal fibroblasts or lymphoblasts. We found that these hybrids, like adult nonerythroid x MEL hybrids, express only β human globin. These results suggest that the derivation of a human parental cell from the fetal stage of development does not necessarily result in activation of the fetal globin program in the hybrids. Our results also support the concept that the hybrids of MEL cells with human fetal erythroid cells express fetal globin because they inherit a "program," already existing in the parental cell, allowing γ-gene expression.

MATERIALS AND METHODS

Cell and fusion procedures. The fetal liver B lymphocyte cell line was established by means of Epstein-Barr (EB) virus transformation of a cell suspension from the liver of an abortus that was 129 gestational days old. The fetal liver fibroblast strain was made from the culture and successive passage of an adherent cell population from the liver of an abortus that was 52 gestational days old. The adult EB virus-transformed B lymphocyte cell line was established from adult peripheral blood mononuclear cells from a normal donor. The tetraploid MEL cell line, clone 179, deficient in adenine phosphoribosyl transferase,[1] was kindly supplied by A. Deisseroth, San Francisco.

MEL cells were fused with the different human nonerythroid cells described earlier (1:10 cell ratio) in the presence of 50% (vol/vol) polyethylene glycol (#P-5402, molecular weight 1,450; Sigma Chemical Co, St Louis) in RPMI-1640 medium at 37 °C. After washing once with RPMI without serum, the cells were resuspended in RPMI with 10% fetal calf serum (FCS) and cultured for 48 hours at 37 °C before selective medium (containing adenine, alanosine, and ouabain) was added.[1] Growth of hybrids was detected 2 to 3 weeks after fusion. Growing hybrids were expanded in nonselective medium and screened as described below.

Screening of hybrid cells for human chromosome 11. Monoclonal antibody 53/6[5] has a strong affinity to a cell surface antigen whose genetic determinants reside on chromosome 11. This antibody was used for screening and enrichment of human chromosome 11-bearing cells in hybrid population with the following live cell immunofluorescence assay and fluorescence-activated cell sorting (FACS).

Hybrid cells were treated with 53/6 hybridoma culture supernatant for 15 minutes at 4 °C. After washing once with cold PBS (phosphate-buffered saline containing 1% FCS and 2 mmol/L sodium azide), they were incubated for another 15 minutes in fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse IgG (Tago, Inc, Burlingame, CA). Cells were again washed and resuspended at high density in PBS containing 1 μg/mL propidium iodide. The percentage of positive cells was estimated by observing wet-mount preparations under a fluorescence microscope. Positive cells appeared lightly speckled on their surfaces; dead cells and debris were bright red. Negative cells were invisible in fluorescent light but could be counted by switching to bright field conditions.

Hybrid populations with less than 20% positive cells with 53/6 were enriched with FACS as previously described.[5] For hybrids with more than 20% 53/6-positive cells, indirect panning[6] was used for enrichment instead of cell sorting using FACS.

Preparations of hybrid cells expressing γ- and β-chains were evaluated in hybrid populations enriched to 80% to 100% positivity with 53/6 by FACS or panning and induced for three to four days with HMBA and hemin.[7] For detection of human globin expression

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Table 1. Hybrids Produced by Fusing MEL Cells With Various Types of Human Cells

Human Parental Cell	Total Generated	Containing Human Chromosome 11
Fetal lymphoid	65	11
Fetal fibroblasts	53	4
Adult lymphoid	57	7
Fetal liver	34	2

by immunofluorescence, cells were fixed and doubly stained with anti-γ- and anti-β-chain monoclonal antibodies,[8] the former conjugated with FITC, the latter with rhodamine, as previously described.[9,10]

Human globin chain synthesis was evaluated by isoelectric focusing[11] after overnight labeling with ^{3}H-leucine of cells that had been induced for three to four days with HMBA and hemin. RNA was prepared[12] from cells induced for three days. RNA was annealed to γ- and β-probes as described by Ley et al.[13] Unprotected sequences were digested by S1 nuclease, and protected sequences were separated by electrophoresis.

RESULTS

Detection and maintenance of human chromosome 11-containing hybrids. Fusion of MEL cells with lymphoblasts from a 129-day-old fetus generated 65 hybrids; a fusion of MEL cells with fibroblasts from a 52-day-old fetus generated a total of 53 hybrids. Fusions of MEL cells with adult lymphoid cells and fetal liver cells served as controls.

The hybrids were first stained with McAb 53/6 two to three weeks after fusion, when they were composed of about 10^6 cells. At this stage the greatest majority of the hybrids contained less than 1:1,000 53/6-positive cells; such hybrids were considered as 53/6 negative. Eleven of the 65 MEL x fetal lymphoid and four of the 53 MEL x fetal fibroblast hybrids were 53/6 positive (Table 1).

Figure 1 shows the proportion of chromosome 11-containing cells at the time of detection of the positive hybrids. Striking differences in frequencies can be noted. In two hybrids, 80% of the cells were positive when these hybrids were stained with 53/6 seventeen to eighteen days after fusion. In other hybrids ascertained at the same time only 1% of the cells retained human chromosome 11.

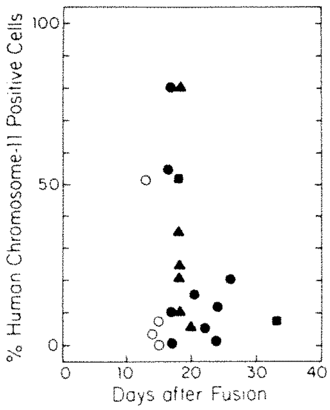

Fig 1. MEL x human cell hybrids containing human chromosome 11. Frequency of chromosome 11-positive cells at the time of ascertainment of hybrids. ● MEL x fetal lymphoid; ○ MEL x fetal fibroblasts; ▲ MEL x adult lymphoid; ■ MEL x fetal liver erythroid cells.

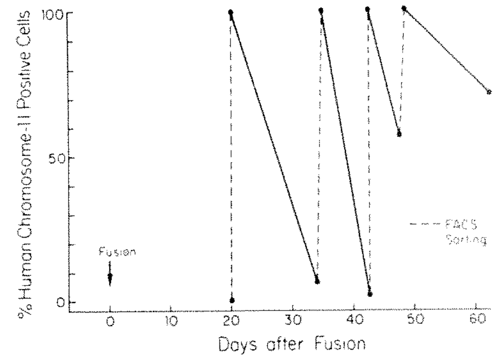

Fig 2. Maintenance of human chromosome 11-containing hybrids in culture by cell sorting. Note that a population of 100% positive cells is generated after FACS sorting of populations containing 0.5% to 5% positive cells.

Even hybrids with low frequencies of chromosome 11-retaining cells could be used in our studies, since the 53/6-positive cells could be sorted by FACS; hence, the chromosome 11-containing hybrids could be maintained in culture in the absence of a selective culture system (Fig 2). Maintenance of such hybrids in culture required frequent monitoring of the frequency of 53/6-positive cells and either sorting using FACS (Fig 2) or panning.

The measurements of 53/6-positive cells at various culture intervals provided information on the rate of loss of chromosome 11 by these hybrids. Certain hybrids lose chromosome 11 at a high rate (Fig 3A), while the rate of loss of chromosome 11 by other hybrids is retarded (Fig 3B).

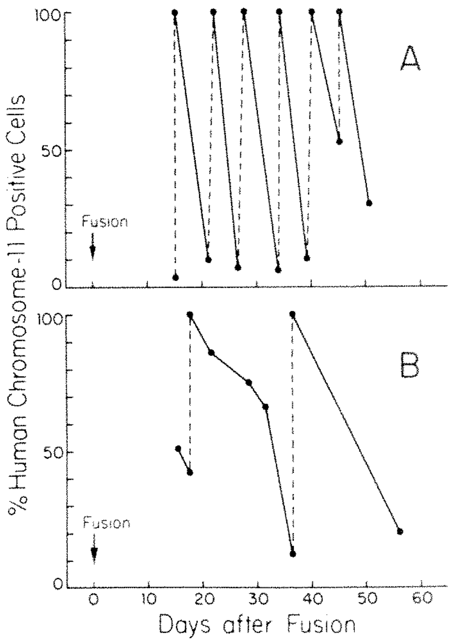

Fig 3. Maintenance of chromosome 11-containing cells in culture. Findings in two hybrids (A and B) showing different rates of loss of human chromosome 11. Broken lines represent FACS sorting.

Because we knew the percentage of cells containing human chromosome 11 at various culture days, we could calculate the per-day rate of loss of human chromosome 11. For example, six days after the first sorting of the hybrid of Fig 3A, 90% of the cells have lost their chromosome 11, corresponding to a loss of 15% per day. In contrast, 20 days after the second sorting of the hybrid of Fig 3B, 80% of the cells have lost their chromosome 11, corresponding to a loss of 4% per day. Data from sequential studies of several hybrids are summarized in Fig 4. There appears to be a decline in the rate of loss of chromosome 11 as culture time passes. Also, these data suggest that there exist differences in the rate of loss of chromosome 11 between lymphoid and fibroblastic hybrids.

Expression of human globins. Globin phenotypes were first assessed with anti–γ- and anti–β-chain McAbs, the former conjugated to FITC, the latter to rhodamine. Hybrid cell populations that were enriched to chromosome 11-positive cells (by panning or FACS) were tested for globin expression. Of the 11 MEL x fetal lymphoid hybrids, nine expressed human globins. All four MEL x fetal fibroblast hybrids expressed human globins.

In the absence of induction by HMBA and hemin, only rare (1:500 to 1:1,000) cells expressed human globin. In contrast, 10% to about 60% of the cells were positive for human globins after induction. All human globin-expressing cells were human β-globin positive (Fig 5). No positive cells were observed after labeling with anti–γ-chain McAbs. Data from measurement of β-positive or γ-positive cells in several hybrids are summarized in Fig 6. It is apparent that the MEL x adult nonerythroid cell hybrids fail to show cells containing γ-globin, but they contain β-globin–positive cells. In contrast, the MEL x fetal liver hybrids express γ-globin consistently. RNA was prepared from hybrids after enrichment to chromosome 11-positive cells by FACS or panning. The RNA was annealed to the γ- and β-probes described by Ley et al,[13] unprotected sequences were digested by S1 nuclease, and protected sequences were separated by electrophoresis. As shown in Fig 7, only β-mRNA can be detected in the MEL x fetal nonerythroid hybrids. Hybrids were incubated with ^{3}H-leucine, the cells were lysed, and the lysates were used for globin chain isoelectric focusing. Only β-chains could be detected by this method in agreement with the results of immunofluorescence and S1 nuclease mapping.

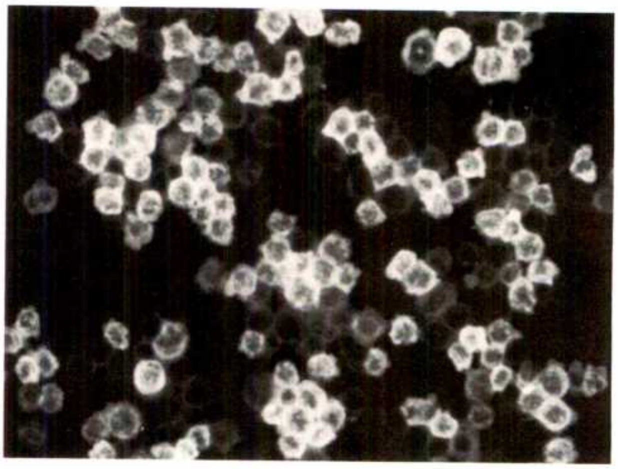

Fig 5. Fluorescent antibody labeling of MEL x human fetal fibroblast cell hybrid. Staining with anti–β-chain McAb conjugated with FITC.

DISCUSSION

Somatic cell hybridization has been a useful tool for studying the control of gene activity in development and differentiation.[14,15] The first application of the approach to the human globin system was done by Deisseroth and Hendrick,[1] who observed that the human globin genes continued to be active in hybrids produced by fusion of MEL cells with human adult bone marrow cells. The availability of a selective marker (APRT) on chromosome 16 (containing the human α-globin locus) facilitated studies of the control of this locus using somatic cell hybridization.[1] In contrast, there

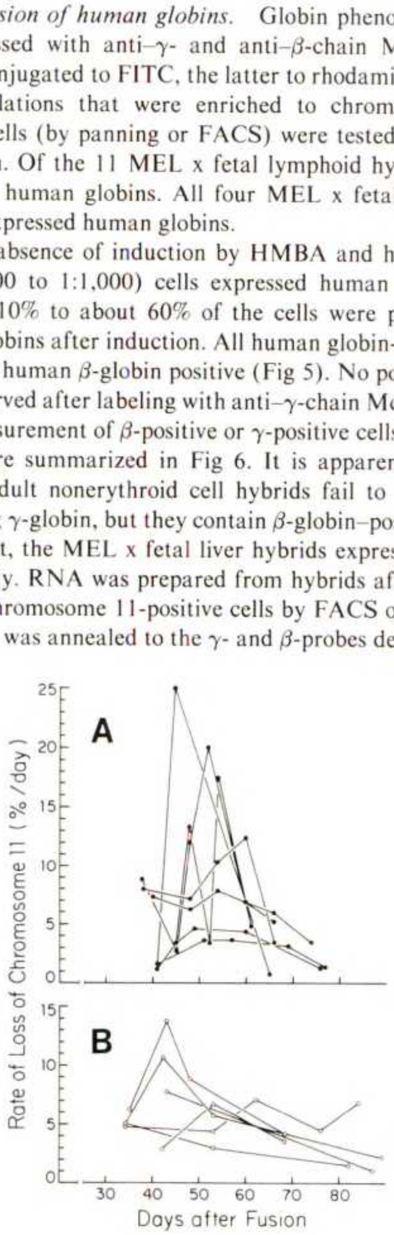

Fig 4. Relationship between the rate of loss of human chromosome 11 (calculated as described in Results) and culture time. (A) MEL x fetal lymphoid hybrids. (B) MEL x fetal fibroblastic hybrids.

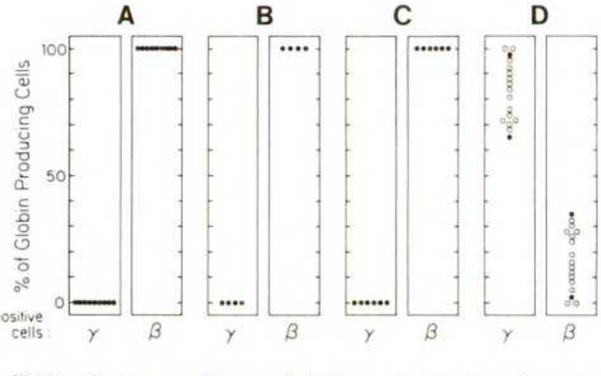

Fig 6. Frequency of human β-globin–positive cells and human γ-globin–positive cells in (A) MEL x fetal lymphoid; (B) MEL x fetal fibroblasts; (C) MEL x adult lymphoid; and (D) MEL x fetal liver erythroid hybrids. ● data from the present study, ○ from reference 4.

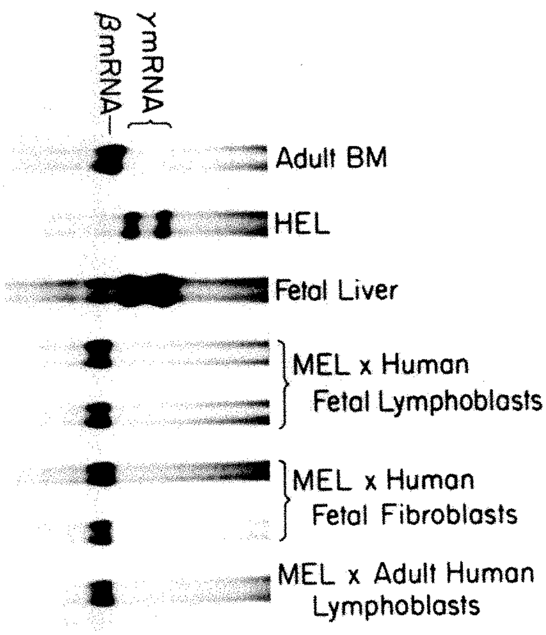
γmRNA
βmRNA
Adult BM
HEL
Fetal Liver
MEL x Human Fetal Lymphoblasts
MEL x Human Fetal Fibroblasts
MEL x Adult Human Lymphoblasts

Fig 7. S1 nuclease mapping of RNA from MEL x fetal nonerythroid cell hybrids and controls. Total cellular RNA was cohybridized to the β or γ probes. In the hybrids, only the mRNA fragment protected by the β-globin probe was detected.

are no markers for chromosome 11, on which the genes of the β-globin cluster are located. Despite this absence, several experiments yielding important information were done. Willing et al[3] fused MEL cells with human nonerythroid cells and found activation, in these hybrids, of the developmentally dormant β-locus of the human fibroblast. Most interestingly, the β- but not the γ-genes of this locus were activated.[3] The absence of γ expression in these nonerythroid x MEL hybrids could be explained by several alternatives. One is that the MEL cells contain an inducer of β-gene expression and lack an inducer of γ-gene expression. A second possibility is that the MEL cells contain an inhibitor of γ-gene expression. Another interpretation is that the adult developmental origin of the human parental cells is responsible for the expression of adult but not fetal human globin genes in the hybrids.

Subsequent studies showed that although the γ-globin genes are not expressed in hybrids of MEL cells with adult nonerythroid cells, the γ-genes are expressed when fetal cells are fused with MEL cells.[4] These results were explained by assuming that the parental human fetal erythroid cells transmitted to the hybrid the information needed for expression of the γ-globin genes. This information could be related to the "program" of the human parental cell, ie, its potential to express the γ-globin genes; alternatively, it could be related to developmental time per se, ie, the origin of the human parental cells from the fetal stage of the human ontogeny. The experiments we describe here were done in order to test the latter possibility. We found that fetal fibroblasts or fetal lymphoblasts fused with MEL cells produce hybrids that express the adult human globin program and fail to express fetal globin. These results support the concept that the γ expression in fetal erythroid x MEL hybrids reflects the program of the human parental cell rather than its origin from the fetus. γ-Globin expression has been detected in hybrids of MEL cells with the human erythroleukemia line HEL,[16] a line that expresses γ-globin. As in the case of fetal erythroid x MEL hybrids, one can conclude that the MEL x HEL hybrids express γ-globin because they inherit from the parental HEL cells a "program" allowing γ expression.

The expression of LDH-A, an enzyme whose locus is on human chromosome 11, has been used as a marker of human chromosome 11-containing hybrids.[3,16] The use of monoclonal antibody 53/6 for detection of hybrids containing human chromosome 11 offers a significant advantage over the LDH-A approach, since it allows ascertainment even of those hybrids that contain a small number of chromosome 11-positive cells. Furthermore, since the hybrid cells preserve their viability after binding of the antibody, it is possible to sort the chromosome 11-positive cells by FACS or purify them by panning. Hence, hybrids that contain even 1:1,000 chromosome 11-positive cells could be used for our studies. In the absence of culture conditions allowing for selection of chromosome 11-containing cells, the hybrids containing these cells are transient because of the continuous random segregation of human chromosome 11. As we have shown here, the use of a constitutive cell surface marker to detect the chromosome 11-positive cells allows rescue of these hybrids by sorting, or panning, it permits continuous growth of chromosome 11-retaining hybrids, and it allows accumulation of the large numbers of cells (10^7 to 10^8 cells) required for certain molecular analysis (such as studies of chromatin). The immunochemical detection of constitutively expressed cell surface determinants can thus be used, in the absence of selective culture systems, in the analysis of the control of human genes through the approach of somatic cell hybridization.

REFERENCES

1. Deisseroth A, Hendrick D: Human α-globin expression following chromosomal dependent gene transfer into mouse erythroleukemia cells. Cell 15:55, 1978

2. Pyati J, Kucherlapati RS, Skoultchi AI: Activation of human β-globin genes from nonerythroid cells by fusion with murine erythroleukemia cells. Proc Natl Acad Sci USA 77:3435, 1980

3. Willing MC, Nienhuis AW, Anderson WF: Selective activation of human β but not γ globin gene in human fibroblast x mouse erythroleukemia cell hybrids. Nature 277:534, 1979

4. Papayannopoulou T, Brice M, Stamatoyannopoulos G: Analysis of human hemoglobin switching in MEL x human fetal erythroid cell hybrids. Cell 46:469, 1986

5. Papayannopoulou T, Brice M, Yokochi T, et al: Anti-HEL cell monoclonal antibodies recognize determinants that are also present in hematopoietic progenitors. Blood 63:326, 1984

6. Greenberg PL, Baker S, Link M, et al: Immunologic selection of hematopoietic precursor cells utilizing antibody-mediated plate binding ("panning"). Blood 65:190, 1985

7. Marks PA, Rifkind RA: Erythroleukemic differentiation. Ann Rev Biochem 117:419, 1978

8. Stamatoyannopoulos G, Farquhar M, Lindsley D, et al: Monoclonal antibodies specific for globin chains. Blood 61:530, 1983

9. Crawford TB, McGuire TC, Hensen JB: Detection of equine infectious anemia virus in vitro by immunofluorescence. Arch Ges Cirusforsch 34:332, 1971

10. Nairn RC (ed): Fluorescent Protein Tracing. Edinburgh, Livingstone, 1969

11. Righetti RG, Gianazza E, Gianni AM, et al: Human globin chain separation by isoelectric focusing. J Biochem Biophys Methods 1:45, 1979

12. Kantor JA, Turner PH, Nienhuis AW: Beta thalassemia: Mutations which affect processing of the β-globin mRNA precursor. Cell 21:149, 1980

13. Ley TJ, Anagnou NP, Pepe G, et al: RNA processing errors in patients with β-thalassemia. Proc Natl Acad Sci USA 79:4775, 1982

14. Harris H: Cell Fusion. Cambridge, MA, Harvard University, 1970

15. Ephrussi B: Hybridization of Somatic Cells. Princeton, NJ, Princeton University, 1974

16. Papayannopoulou Th, Lindsley D, Kurachi S, et al: Adult and fetal human globin genes are expressed following chromosomal transfer into MEL cells. Proc Natl Acad Sci USA 82:780, 1985

The British Form of Hereditary Persistence of Fetal Hemoglobin Results From a Single Base Mutation Adjacent to an S1 Hypersensitive Site 5′ to the $^A\gamma$ Globin Gene

By V.E. Tate, W.G. Wood, and D.J. Weatherall

The $^G\gamma$ and $^A\gamma$ genes of an individual homozygous for the British form of $^A\gamma$ nondeletion hereditary persistence of fetal hemoglobin have been cloned and partially sequenced. The $^G\gamma$ gene was normal, but the $^A\gamma$ gene was found to have a single base change (T → C) at −198 bp relative to the cap site. Supercoiled plasmids containing normal γ-genes or the mutant $^A\gamma$-gene displayed an S1-hypersensitive site immediately 5′ to the base change.
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THE HUMAN β-like globin genes are arranged in a single cluster on the short arm of chromosome 11 in their order of expression during development; 5′ ϵ-$^G\gamma$-$^A\gamma$-δ-β-3′.[1,2] During much of fetal life the $^G\gamma$- and $^A\gamma$-genes account for >90% of globin chain production directed by this cluster, but during the perinatal period this diminishes to be reciprocally replaced by adult β-chain and a small amount of δ-chain. Although developmental changes in the structure of the complex have been described,[3,4] little is known about the regulation of the differential expression of its constituent genes. One approach to this question is to analyze the molecular basis for conditions in which there is a genetic defect in the pattern of gene switching in the cluster.

Conditions in which fetal hemoglobin production in adult life exceeds the normal level of <1%, in the absence of any major hematologic changes, are known collectively as hereditary persistence of fetal hemoglobin (HPFH). They are phenotypically heterogeneous, both in the amounts of Hb F ($\alpha_2\gamma_2$) produced and in the ratio of $^G\gamma$-and $^A\gamma$-chains that it contains. Molecular analysis of these conditions has shown that the majority are due to gene deletions removing variable amounts from the 3′ end of the complex, usually including the δ-and β-genes.[1,2] However, in several cases gene mapping has shown no gross alteration of the structure of the complex in heterozygotes, despite the production of 10% to 20% Hb F in adult life. In these nondeletion HPFH disorders, γ-chain production is almost entirely derived from only one of the two γ-genes, and in each case there is good evidence that the β-gene on the affected chromosome is also expressed. Cloning and sequencing of the overexpressed γ-gene have revealed single base substitutions in several of these disorders in the region 5′ to the transcription initiation point, at $^G\gamma$-202 in $^G\gamma\beta^+$ HPFH,[5] $^A\gamma$-117 in Greek HPFH,[6,7] and $^A\gamma$-196 in an Italian case.[8] This region is believed to be of importance in transcriptional regulation, although as yet there is no direct evidence that these substitutions are responsible for the increased γ-chain production.

The British type of nondeletion HPFH has been described in a single large family, which is unique in that it contains the only described homozygotes for one of these conditions and that the phenotypic effects of the disorder have been studied not just in adults, but also from birth through early childhood.[9,10] We have cloned and sequenced both the $^G\gamma$-and the $^A\gamma$-genes from one of the homozygotes; the only difference from normal is a single base substitution at position −198 of the adult-expressed $^A\gamma$-gene.

MATERIALS AND METHODS

Details of the British family here have been reported elsewhere.[9,10] Blood was obtained from one of the homozygotes (III.5) who has 20% Hb F, mainly of the $^A\gamma$-type. High-molecular-weight DNA prepared from whole frozen blood of individual III.5 was digested to completion with *Bgl*II (N.E. Biolabs, Beverly, MA) according to the manufacturer's instructions, and the DNA was enriched for the 13-kb size range by 5% to 20% isokinetic sucrose gradient centrifugation. Appropriately sized DNA was cloned into the *Bam*HI site of EMBL 3, and γ-gene–containing phage were selected by hybridization with the nick translated 3.3-kb *Hind*III fragment from a plasmid subclone containing a normal $^A\gamma$-gene. A 2.8-kb *Bst*XI–*Hind*III $^G\gamma$-fragment (coordinates 1299 through 4112 of Shen et al[11]) and a 3.3-kb *Hind*III $^A\gamma$-fragment (coordinates 5714 through 9012) were subcloned separately into the *Hind*III site of pBR322, and the inserted DNA was sequenced by the method of Maxam and Gilbert[12] on both strands.

S1 nuclease analysis. Digestions of the $^A\gamma$ and $^G\gamma$ subclones were performed at a DNA concentration of 25 μg/mL, using 120 units S1 nuclease (Sigma Chemical Co, St Louis) per milliliter, in 30 mmol/L Na acetate pH 4.6, 3 mmol/L $ZnCl_2$, 0.5 mmol/L EDTA, 0.25 mol/L NaCl at 37 °C. Under these conditions the majority of DNA molecules were nicked once to produce open circles, but not linearized, as judged by ethidium bromide-stained agarose gel electrophoresis. Long periods of digestion were avoided to prevent progressive nuclease digestion away from the primary cut site. Plasmids were then cut with *Stu*I and labeled, on either the antisense or sense strands using terminal transferase (Amersham, UK) and T4 polynucleotide kinase (NEN Research Products, DuPont UK), respectively, making it possible to measure the distance of any S1 nuclease-hypersensitive site (HSS) from the labeled site. The plasmids were redigested with *Rsa*I, which cuts 24 nucleotides to the 5′ side of the *Stu*I site and 200 bp 3′ of the poly A addition site of the $^A\gamma$-gene. The products of these reactions were separated on denaturing polyacrylamide gels. Control plasmids were treated in the same way, but omitting the S1 enzyme, and then subjected to Maxam and Gilbert sequencing to act both as position markers and as controls.

All other enzyme reactions and cloning and sequencing techniques used protocols as laid out by Maniatis et al.[13]

RESULTS

Full details of the phenotypic effects of British HPFH have been described previously.[9,10] Homozygotes are clini-

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cally and hematologically normal except for increased Hb F levels of 18% to 21%, in which the proportion of $^{A}\gamma$-chains is 90% to 95%. Levels of Hb F in heterozygotes are lower, 3.5% to 10%, with a similar $^{G}\gamma$-$^{A}\gamma$ ratio but a markedly uneven intercellular distribution. Restriction enzyme mapping of the homozygotes has shown no abnormalities within the β-globin gene cluster[14]; haplotype analysis showed homozygosity at all the polymorphic restriction enzyme sites in the β-globin gene cluster.[15]

Sequence of the $^{G}\gamma$-gene. The subcloned $^{G}\gamma$-gene was sequenced from position −325 bp to +503 bp relative to the cap site (+1). The sequence was almost identical to that described previously as the normal chromosome B allele (J. Slightom, personal communication, 1985)[11,16]; the only differences were a T → C transition at −158 and a C → T transition at +210, both of which have been reported in other normal B-type alleles.[5]

Sequence of the $^{A}\gamma$-gene. A total of 673 bp of the $^{A}\gamma$ gene were sequenced, from −383 bp to +290 bp, and the $^{A}\gamma$ sequence was also that of a typical B-type chromosome, but including the −158 T → C and +210 C → T changes noted in the $^{G}\gamma$-gene. One additional change was also observed, a T → C transition at position −198 (Fig 1), in a similar but different position to those seen in other cases of nondeletion HPFH.

S1 nuclease sensitivity. Among the changes known to be associated with gene expression is an increased susceptibility of chromatin at the 5′ end of the gene to digestion by a variety of nucleases. In some cases these regions may also be

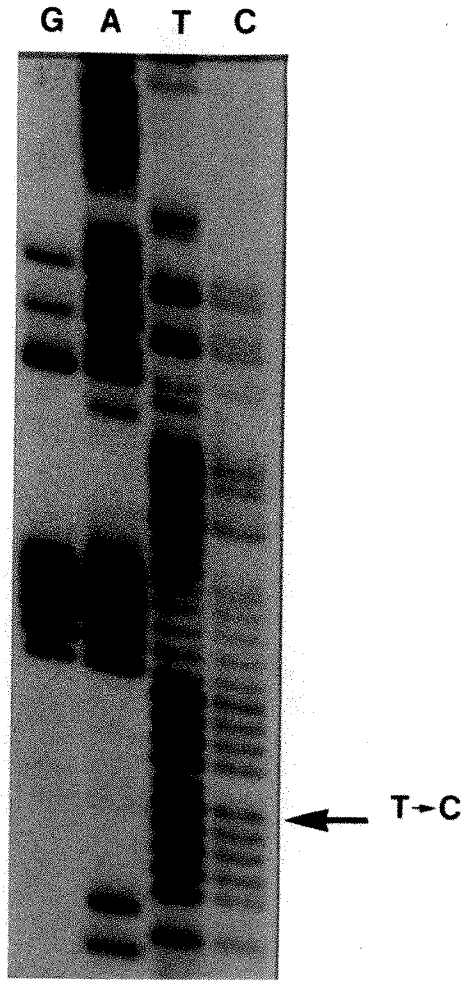

Fig 1. The DNA sequencing gel of part of the British HPFH $^{A}\gamma$-gene. The base change at position −198 is indicated.

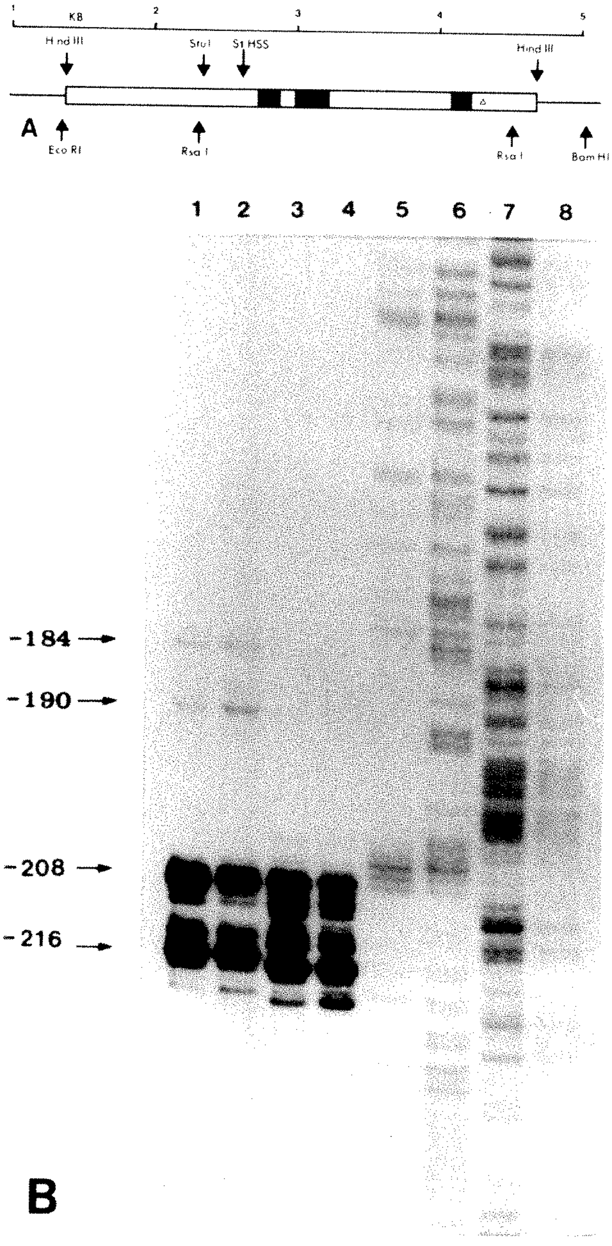

Fig 2. (A) Restriction enzyme sites used in mapping the S1 nuclease hypersensitive site (S1 HSS) in the $^{A}\gamma$-gene. The shaded boxes are the coding regions and the triangle marks the poly A addition site. The sense or antisense strands were labeled at the *Stu*1 site, after S1 digestion, to enable position determination of the HSS, as detailed in Materials and Methods. (B) Major S1 hypersensitive sites in plasmids containing normal (lanes 1 and 3) and British HPFH (lanes 2 and 4) $^{A}\gamma$-genes, labeled on antisense (lanes 1 and 2) and sense (lanes 3 and 4) strands. Lanes 5, 6, 7, 8 are G, A, T, C sequencing products of British HPFH $^{A}\gamma$-gene labeled on the sense strand at the *Stu*1 site.

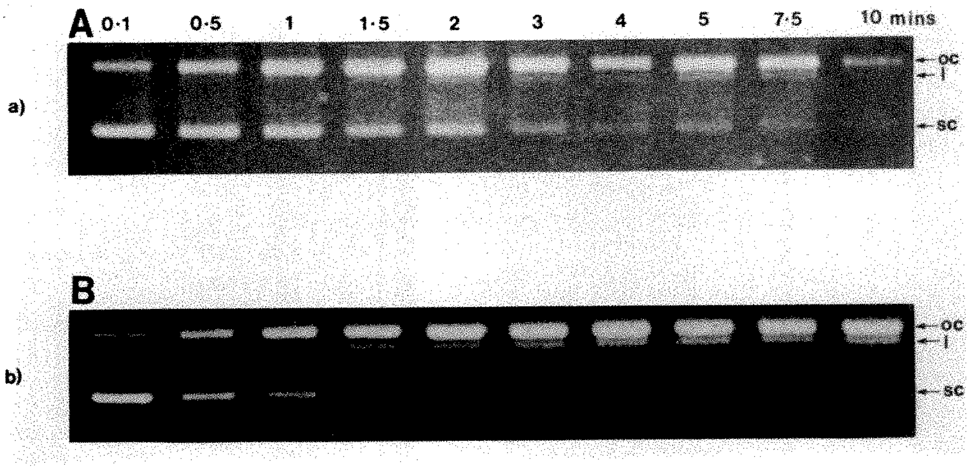

Fig 3. Time course of conversion of plasmids containing normal (A) and British HPFH (B) $^{A}\gamma$-genes from supercoils (sc) to open circles (oc) or linear (l) molecules.

demonstrable as S1 nuclease HSS in supercoiled plasmid DNA.[17] Therefore, the S1 nuclease HSS in plasmids containing normal $^{G}\gamma$- and $^{A}\gamma$-genes were mapped and the results compared with the $^{A}\gamma$-gene isolated from the British HPFH homozygote.

Figure 2A shows the key sites used in the S1 nuclease analysis, while Fig 2B shows that the strongest S1 HSS upstream of the $^{A}\gamma$-promoter lies between nucleotides −204 and −220, immediately to the 5′ side of the region in which the mutations in the British HPFH, and other forms of nondeletion HPFH, occur. There is no difference in the position or architecture of this S1 HSS between the normal and British HPFH $^{A}\gamma$-genes. The only change that has been noted is that plasmids containing the British HPFH promotor regions appear to be converted to nicked circles by S1 at approximately twice the rate of the normal control (Fig 3). The S1 HSS is essentially symmetrical on sense and antisense strands and appears to show two hypersensitive regions within the overall HSS. This is shown diagrammatically in Fig 4. Plasmids containing the $^{G}\gamma$-promoter region show an identical S1 HSS, whereas $^{G}\gamma$- or $^{A}\gamma$-containing plasmids, linearized before S1 treatment, do not show sites of S1 sensitivity (data not shown). Therefore, S1 hypersensitivity is a property of DNA structure and requires torsional stress for its induction. There are additional, much weaker, sites of S1 hypersensitivity, on the antisense strand only and centering around nucleotides −190, −184, and −124 found in both the normal and the British HPFH $^{A}\gamma$-genes (Fig 2B), as well as one site at −420 on the $^{A}\gamma$-genes but not on $^{G}\gamma$ (data not shown).

DISCUSSION

Cloning and sequencing of the γ-genes from a homozygote for British HPFH has demonstrated, as the only unusual feature, a single base substitution (T → C) at position −198, 5′ to the cap site of the overexpressed $^{A}\gamma$-gene. This observation, together with three single base substitutions described recently in similar disorders (at $^{A}\gamma$ −117 in Greek HPFH, at $^{A}\gamma$ −196 in an Italian case, and at $^{G}\gamma$ −202 in $^{G}\gamma\beta^{+}$ HPFH) and the lack of similar substitutions in the normal γ-genes,[18,19] makes a causal relationship between the substitution and the phenotypic effect of increased γ-chain production in adult life very likely, even if it has yet to be demonstrated directly.

The region of DNA 5′ to the γ-genes has been implicated in the regulation of γ-gene expression by several lines of evidence. Deletions that remove sequences between −260 and −104 cause a marked reduction of γ-gene transcription in a transient expression assay.[20] The region is marked by upstream transcription initiation sites,[21,22] although the significance of such sites has yet to be established. Digestion of intact nuclei with DNase I has revealed a DNase I HSS 5′ to the expressed γ-genes at ~ −100 in K562 erythroleukemia cells[23] and in human fetal erythroid cells when γ-chain production is fully active, but not in adult cells, where they are largely repressed.[4] Such sites appear, therefore, to play a developmentally related role in regulation in vivo.

Of the nondeletion HPFH disorders, the British family is the best characterized at the phenotypic level.[9,10] Thus, at birth, British HPFH heterozygotes have normal Hb F levels and a normal $^{G}\gamma$-$^{A}\gamma$ chain ratio; were the affected chromosome producing γ-chains at the same ratio as in adult life (>90% $^{A}\gamma$), it would have been readily detectable in the newborn period. Adult homozygotes for British HPFH show no hematologic abnormalities and, in particular, no evidence of imbalanced production of α- versus $\gamma + \beta$-chains. In other words, γ-chain production in these cells does not appear to be additional to a normal level of β-chain production, but to be

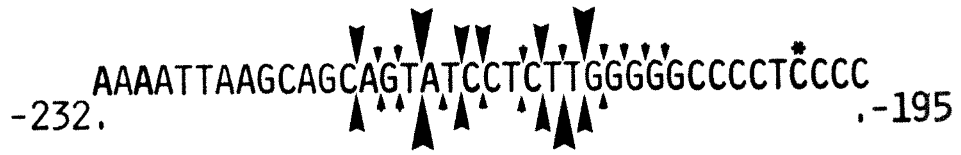

Fig 4. Diagrammatic representation of the S1 HSS 5′ to the promoter of the $^{A}\gamma$-gene. The sizes of the arrows represent the frequency with which a particular site is cut. *T → C transition in British HPFH.

associated with a reciprocal reduction in β-chain output. Furthermore, the intercellular distribution of Hb F is clearly heterogeneous, and in heterozygotes, many cells do not contain detectable amounts of Hb F.[9,10]

If the single base substitutions upstream from the γ-genes are responsible for the increased Hb F production in these disorders, does the phenotypic information provide any clues or place any constraints on the mechanism? The most likely explanation is that the region upstream of the affected gene is involved in the interaction with a trans-acting factor and that the effect of the substitution is to increase the affinity of this sequence for a factor that promotes γ-gene expression or, alternatively, to decrease its affinity for a repressor molecule. It is clear from the deletion HPFH conditions and other disorders that all of the trans-acting factors required for γ-gene expression are present in adult cells. Furthermore, there is evidence that all adult erythroid cells have the potential for Hb F production and that the low level of γ-gene expression in a small proportion of adult red cells (F cells) appears to be controlled stochastically.[24] It is quite plausible, therefore, that the effect of the single base substitution, by altering the affinity for a putative trans-acting factor, would be to increase the normal propensity of adult cells to make a little Hb F rather than by activating a dormant gene in a novel manner. This notion is consistent with the variable Hb F levels in heterozygotes and the heterocellular distribution. One would not expect, therefore, any effect during fetal life when γ-gene expression is maximal anyway, presumably as a result of a more profound change in chromatin structure of the globin gene complex. This again is consistent with the observations on the phenotype in the cord blood of heterozygotes.[10]

The reciprocal relationship between γ- and β-chain synthesis in the affected cells is likely to be a direct corollary of γ-gene transcription. Although we have no evidence in the British HPFH family that the reciprocity is a cis-active effect, that does appear to be the case in ${}^{G}\gamma\beta^{+}$ HPFH when there is a β^{S} or β^{C} marker on the other chromosome; any competition between γ- and β-genes for trans-acting factors should affect both β-genes equally. Models in which reciprocity in γ- and β-gene expression could occur as a result of a 5′ → 3′ polarity in the propagation of altered chromatin structure during erythroid cell maturation have been discussed previously.[14,25] A delay in this progression as a result of an increased propensity to transcribe the affected γ-gene in adult cells could result in reduced β-gene transcription.

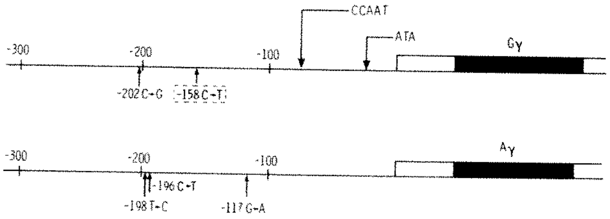

Fig 5. **A summary of the mutations found to date in nondeletion HPFH. The −158 change is represented by a broken box because it appears to be expressed only in adults with sickle cell anemia or β-thalassemia as a relatively high ${}^{G}\gamma$-${}^{A}\gamma$ ratio.[26]**

The four single base substitutions associated with nondeletion HPFH (Fig 5) could represent a single regulatory region spanning at least from −117 to −202, or two separate regions, one based on −117 and the other including −196 through −202. The −117 site lies within the upstream member of a duplicated region of 16 nucleotides, which includes the CCAAT box, a region of known regulatory importance.[1,18] The sites based around −200 are adjacent to a GC-rich region with similarities to regulatory sequences associated with the 21 bp repeat of SV40 virus,[27] herpes virus TK,[28] and an African green monkey gene.[29] These sequences are known to be associated with the binding of a specific protein, SP1.[30] This is also the region that we have demonstrated to contain the S1 HSS site in supercoiled plasmids containing the normal ${}^{G}\gamma$- and ${}^{A}\gamma$-genes.

An S1 HSS has been mapped to a string of G residues (−196 through −180) in the chick adult β-globin gene,[31,32] although cutting is unilateral in this case, and to a homopurine stretch in the region −199 through −207 in human β-globin genes.[33] The γ-globin gene HSS is less homogeneous in that it contains a block of seven pyrimidine residues, followed by five consecutive purine residues, and is bilateral. It will not form any apparent cruciform structure, which could account for its nuclease sensitivity, and therefore the sequence requirement for its structure is unclear. Although the ${}^{A}\gamma$ HSS was unaffected by the base substitution at position −198 in the British HPFH ${}^{A}\gamma$-gene, it is possible that a trans-acting regulatory element interacting with the S1 HSS site might have further contacts in the −200 region that are altered by the substitution. Although we can only speculate about such mechanisms at the moment, it should soon be possible to approach the question of specific protein binding to the region immediately upstream of the γ-genes, greatly facilitated by the availability of cloned material from four different but similar natural mutants.

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REFERENCES

1. Collins FS, Weissman SM: The molecular genetics of human hemoglobin. Prog Nucleic Acids Res Mol Biol 31:315–458, 1984

2. Weatherall DJ, Wood WG, Jones RW, et al: Developmental genetics of human hemoglobin, in Stamatoyannopoulos G, Nienhuis AW (eds): Experimental Approaches for the Study of Haemoglobin Switching. New York, Liss, 1985, pp 3–35

3. Mavilio F, Giampaolo A, Care A, et al: Molecular mechanisms of human hemoglobin switching: Selective undermethylation and expression of globin genes in embryonic, fetal and adult erythroblasts. Proc Natl Acad Sci USA 80:6907–6911, 1983

4. Groudine M, Kohwi-Shigematsu T, Gelinas R, et al: Human fetal to adult hemoglobin switching: Changes in chromatin structure of the β-globin gene locus. Proc Natl Acad Sci USA 80:7551–7555, 1983

5. Collins FS, Stoeckert CJ, Serjeant GR, et al: ${}^{G}\gamma\beta^{+}$ hereditary persistence of fetal hemoglobin: Cosmid cloning and identification of

a specific mutation 5′ to the $^{G}\gamma$ gene. Proc Natl Acad Sci USA 81:4894–4898, 1984

6. Collins FS, Metherall JE, Yamakawa M, et al: A point mutation in the $^{A}\gamma$-globin gene promoter in Greek hereditary persistence of fetal haemoglobin. Nature 313:325–326, 1985

7. Gelinas R, Endlich B, Pfeiffer C, et al: G to A substitution in the distal CCAAT box of the $^{A}\gamma$-globin gene in Greek hereditary persistence of fetal haemoglobin. Nature 313:323–325, 1985

8. Giglioni B, Casini C, Mantovani R, et al: A molecular study of a family with Greek hereditary persistence of fetal hemoglobin and β-thalassemia. EMBO J 3:2641–2645, 1984

9. Weatherall DJ, Cartner R, Clegg JB, et al: A form of hereditary persistence of fetal haemoglobin characterised by uneven cellular distribution of haemoglobin F and the production of haemoglobins A and A_2 in homozygotes. Br J Haematol 29:205–220, 1975

10. Wood WG, Macrae IA, Darbre PD, et al: The British type of non-deletion HPFH: Characterisation of developmental changes *in vivo* and erythroid growth *in vitro*. Br J Haematol 50:401–414, 1982

11. Shen S, Slightom JL, Smithies O: A history of the human fetal globin gene duplication. Cell 26:191–203, 1981

12. Maxam AM, Gilbert W: Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol 65:499–590, 1980

13. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning—A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1982

14. Jones RW, Old JM, Wood WG, et al: Restriction endonuclease maps of the β-like globin gene cluster in the British and Greek forms of HPFH, and for one example of $^{G}\gamma\beta^{+}$ HPFH. Br J Haematol 50:415–422, 1982

15. Old JM, Ayyub H, Wood WG, et al: Linkage analysis of non-deletion hereditary persistence of fetal hemoglobin using DNA polymorphisms. Science 215:981–982, 1982

16. Slightom JL, Blechl AE, Smithies O: Human fetal $^{G}\gamma$- and $^{A}\gamma$-globin genes: Complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. Cell 21:627–638, 1980

17. Weintraub H: A dominant role for DNA secondary structure in forming hypersensitive structures in chromatin. Cell 32:1191–1203, 1983

18. Collins FS, Boehm CD, Waber PG, et al: Concordance of a point mutation 5′ to the $^{G}\gamma$ globin gene with $^{G}\gamma\beta^{+}$ hereditary persistence of fetal hemoglobin in the Black population. Blood 64:1292, 1984

19. Waber PG, Bender MA, Gelinas RE, et al: Concordance of a point mutation 5′ to the $^{A}\gamma$-globin gene with $^{A}\gamma\beta^{+}$ hereditary persistence of fetal hemoglobin in Greeks. Blood 67:551–554, 1986

20. Anagnou NP, Karlsson S, Moulton AD, et al: Promoter sequences required for function of the human γ globin gene in erythroid cells. EMBO J 5:121–126, 1986

21. Grindlay GJ, Lanyon WG, Allan M, et al: Alternative sites of transcription initiation upstream of the canonical cap site in human γ-globin and β-globin genes. Nucleic Acids Res 12:1811–1820, 1984

22. Partington G, Yarwood N, Rutherford TR: Human globin gene transcription in injected Xenopus oocytes: Enhancement by sodium butyrate. EMBO J 3:2787–2792, 1984

23. Lachman HM, Mears JG: DNase I hypersensitivity in the γ globin gene locus of K562 cells. Nucleic Acids Res 11:6065–6077, 1983

24. Stamatoyannopoulos G, Kurnit D, Papayannopoulou T: Stochastic expression of fetal hemoglobin in adult erythroid cells. Proc Natl Acad Sci USA 78:7005–7009, 1981

25. Wood WG, Jones RW: Erythropoiesis and hemoglobin production: A unifying model involving sequential gene activation, in Stamatoyannopoulos G, Nienhius AW (eds): Hemoglobin in Development and Differentiation. New York, Liss, 1981 p 243

26. Gilman JG, Huisman THJ: DNA sequence variation associated with elevated fetal $^{G}\gamma$ globin production. Blood 66:783–787, 1985

27. Dynan WS, Tjian R: The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35:79–87, 1983

28. McKnight SL, Kingsbury RC, Spence A, et al: The distal transcription signals of the herpes virus tk gene share a common hexanucleotide control sequence. Cell 37:253–262, 1984

29. Saffer JD, Singer MF: Transcription from SV40-like monkey DNA sequences. Nucleic Acids Res 12:4769–4788, 1984

30. Dynan WS, Tjian R: Isolation of transcription factors that discriminate between different promoters recognised by RNA polymerase II. Cell 32:669–680, 1983

31. Schon E, Evans T, Welsh J, et al: Conformation of promoter DNA: Fine mapping of S1-hypersensitive sites. Cell 35:837–848, 1983

32. Nickol JM, Felsenfeld G: DNA conformation at the 5′ end of the chicken adult β globin gene. Cell 35:467–477, 1983

33. Evan T, Schon E, Gora-Maslak G, et al: S1-hypersensitive sites in eukaryotic promoter regions. Nucleic Acids Res 12:8043–8058, 1984

CONCISE REPORT

β°-Thalassemia in Association With a γ-Globin Gene Quadruplication

By K.G. Yang, J.Z. Liu, F. Kutlar, A. Kutlar, C. Altay, A. Gurgey, and T.H.J. Huisman

We have studied the hematology, hemoglobin composition, and globin gene arrangements in one young Turkish boy with a β°-thalassemia homozygosity and in 11 of his relatives. Evidence is presented that the chromosome with the β°-thalassemia determinant carries a γ-globin gene quadruplication, perhaps in a -$^{G}\gamma$-$^{G}\gamma$-$^{G}\gamma$-$^{A}\gamma$-gene arrangement. The eight γ-globin genes in this patient produced $^{G}\gamma$ and $^{A}\gamma$ chains in a 95 to 5 ratio, and nearly 99% of the patient's hemoglobin was of the fetal type. The clinical condition resembled that of a thalassemia intermedia. HbF levels in eight β-thalassemia heterozygotes varied between 0.5 and 4.2% and the percentages of $^{G}\gamma$ in this HbF averaged at 87% or 95%; this level is to some extent related to the haplotype of the normal chromosome. All subjects carried four α-globin genes; a new *Bgl*II polymorphism was observed within the $\psi\alpha$-globin gene.

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RECENTLY WE HAD the opportunity to study several members of a Turkish family with β°-thalassemia, including a young boy who was homozygous for this condition. An interesting observation was that the chromosome with the β°-thalassemia determinant carried four γ-globin genes (ϵ-γ-γ-γ-γ-$\psi\beta$-δ-β^{Th}), which resulted in the synthesis of hemoglobin F (HbF) with mainly $^{G}\gamma$ chains. This report provides details of the study.

MATERIALS AND METHODS

Blood samples were collected in vacutainers with EDTA as anticoagulant, and shipped in wet ice by air from Turkey to Augusta, GA. Hematological data were obtained using an automated cell counter.

Hb analyses involved cellulose acetate electrophoresis at pH 8.9,[1] HbA_2 quantitation by microcolumn chromatography,[2] HbF quantitation by alkali denaturation [$\%F_{AD}$],[3] by a more accurate chemical procedure [$\%F_{Ile}$],[4] and by cation exchange high-performance liquid chromatography (HPLC).[5] HbF was isolated by DEAE-cellulose chromatography[6] and its chain composition determined by reverse-phase HPLC[7].

DNA was isolated from peripheral white cells of the propositus and 11 relatives by the method of Poncz et al.[8] These samples were digested with the restriction enzymes *Hinc*II, *Xmn*I, *Bgl*II, *Bcl*I, *Hind*III, *Ava*II, *Hpa*I, *Sph*I, and *Bam*HI, and hybridization was with one of the following probes: ϵ, γIVS-II, inter-γ, $\psi\beta$, and βIVS-II. Details about probes and methodology are given elsewhere.[9-11] In some instances, the X-ray film was scanned to evaluate the relative intensities of some of the bands. The number of α-globin genes was determined through hybridization of *Bgl*II, *Bam*HI, *Hpa*I, and *Eco*RI digests with the α and ζ probes following methodology routinely in use in our laboratory.[12,13]

RESULTS

The family. The propositus was a 10-year-old boy who was known to have a homozygous β°-thalassemia. He had no facial changes suggesting a severe Cooley's anemia and did not require blood transfusions. Growth was greatly retarded with weight and height below the third percentile. The liver and spleen were 10 cm and 20 cm, respectively, below their costal margins. A recent splenectomy greatly improved the propositus' clinical condition. He belongs to a large family in Gaziantep, in southeastern Turkey. From the abbreviated pedigree of Fig 1 a considerable consanguinity is evident; the parents are first cousins because their fathers are brothers, and their mothers, although more distantly, are also related to each other and to their husbands.

Hemoglobin analyses. Hematologic data are given in Table 1. A distinct microcytosis with mild hypochromia was present in all subjects with elevated levels of HbA_2. The propositus (VI-2) had a moderate hypochromic, microcytic anemia with poikilocytosis; HbA_2 was 1.4%, HbF was 98.6%, and HbA was absent. In vitro chain synthesis analyses gave a high α/non-α ratio of 3.13. All β°-thalassemia heterozygotes with elevated HbA_2 levels (4.1% to 5.1%) had slightly elevated levels of HbF (0.5% to 4.2% as $\%F_{Ile}$; 0.7% to 3.8% as $\%F_{AD}$; the correlation between the individual values obtained with the two methods was poor). The γ chain composition of the HbF showed high $^{G}\gamma$ values for the propositus VI-2 (95.3% $^{G}\gamma$) and for the eight β-thalassemia heterozygotes (85.0% to 95.3%).

DNA analyses. Digests of DNA from the propositus (VI-2) with the enzymes *Xmn*I, *Bgl*II, and *Bcl*I contained large fragments which hybridized with the γIVS-II and inter-γ probes; their sizes were consistent with a γ-globin gene quadruplication.[14,15] The patient was identified as a homozygote because normal-sized fragments were not observed (Fig 2). All eight β-thalassemia heterozygotes had the same large fragments in addition to the normal-sized 8-kb *Xmn*I, 13-kb *Bgl*II, and 18-kb *Bcl*I fragments. Haplotyping involved nine restriction sites (*Hinc*II 5' to ϵ; *Xmn*I 5' to $^{G}\gamma$; *Hind*III within $^{G}\gamma$ and $^{A}\gamma$; *Hinc*II within $\psi\beta$ and 3' to it; *Ava*II in β; *Hpa*I and *Bam*HI 3' to β) and showed homozygosity at all sites in the propositus. Figure 2 illustrates the *Hind*III fragments observed in DNA digests of the propositus and his 11 relatives after hybridization with the

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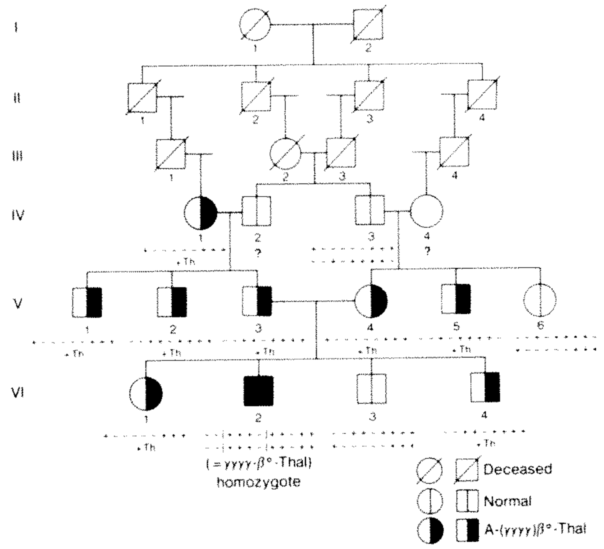

Fig 1. Abbreviated pedigree of Family S with a chromosome with a γ globin gene quadruplication and a β°-thalassemia. Haplotype of the (γγγγ)-β°-thalassemia is listed below the symbol of the propositus VI-2, and the haplotypes of the normal chromosomes under the symbols of the 11 relatives from whom DNA was available.

γIVS-II probe. The propositus had a 7.2-kb fragment only (indicating +/+ at $^G\gamma$) and 3.5- and 2.7-kb fragments in a 1 to 2 ratio, suggesting the presence of this restriction site in two of the three additional γ globin genes. Three of his relatives had a 1:3 ratio and six had a 1:1 ratio, while two normal relatives had the 3.5-kb fragment only (indicating −/− at $^A\gamma$). The haplotype of the (γγγγ)-β°-thalassemia chromosome was [− − (+ + + −)+ + − + +]. Three haplotypes were found for the normal chromosomes: [+ − − − − − + + +] in four β-thalassemia heterozygotes (including the mother) and in three normal relatives; [− − + + − + + + +] in three β-thalassemia heterozygotes (including the father) and in one normal relative, and [− − + − + + + + −] in one β-thalassemia heterozygote and in two normal relatives. Haplotypes for the individual family members are listed in Fig 1. β-Thalassemia heterozygotes with haplotype [− − + + − + + + +] (V-2, V-3, and VI-4) had the high 2.7/3.5-kb *Hind*III fragment ratio of 3, as expected. The number of α-globin genes was four in all members listed in Table 1 and Fig 1, and no ζ deletions were observed. Eight subjects (including the propositus and his parents) had a newly discovered *Bgl*II restriction site within the ψα globin gene; the two brothers of the propositus (VI-3 and VI-4) had this site on both chromosomes.

DISCUSSION

The β°-thalassemia in the propositus is unusual because this homozygous patient never required blood transfusions, despite a rather severe clinical condition marked by a moderate anemia, retarded growth, and severe hepatosplenomegaly. Most likely, the large number of γ-globin genes (eight total) might contribute to a production of γ chains which is probably higher than in a homozygote with the normal number of γ-globin genes. Interestingly, none of the eight β-thalassemia heterozygotes had markedly elevated levels of HbF; the greatly increased production of γ chains apparently depends on the anemic condition of the subject. The specific mutation leading to this type of β°-thalassemia is presently under study.

It is difficult to define the quadruplication of the γ-globin gene in greater detail without data from sequence analyses. Recently, Liu et al[16] identified the crossover area between γ-globin genes in two patients with a γ-globin gene triplication. Both adult subjects, one Yugoslavian[17] and the other Turkish, were heterozygous for a chromosome with one $^A\gamma$- and two $^G\gamma$-globin genes in the -$^G\gamma$-$^G\gamma$-$^A\gamma$-arrangement. The differences between the two conditions were in the crossover region between the first two $^G\gamma$-globin genes; the Yugoslavian patient had a crossover between bases 6591 and 6663,

Table 1. Hematological and Hemoglobin Composition Data for Several Members of the Turkish Family with (γγγγ)-β°-Thalassemia*

Case	Sex/Age	Hb g/dL	PCV 1/1	RBC 10^{12}/L	MCV fL	A_2 %	F_{AD}† %	F_{Be}‡ %	G_γ§ %	A_γ§ %
IV-1	F-55	10.7	.45	5.7	74	4.1	3.8	0.9	88.0	12.0
IV-2	M-65	12.0	.45	4.2	98	2.5	0.4	—	—	—
IV-3	M-70	—	—	—	—	2.6	0.1	—	—	—
V-1	M-34	—	—	—	—	4.5	3.7	4.2	87.2	12.8
V-2	M-18	—	—	—	—	4.8	3.7	4.2	95.1	4.9
V-3	M-36	12.8	.51	7.1	69	4.4	2.5	1.2	95.3	4.7
V-4	F-35	9.7	.41	5.4	72	4.7	3.1	0.8	87.0	13.0
V-5	M-32	—	—	—	—	5.1	0.7	1.8	87.9	12.1
V-6	F-26	—	—	—	—	2.5	0.2	—	—	—
VI-1	F-12	10.4	.41	6.4	68	4.3	1.1	0.5	85.0	15.0
VI-2	M-10	10.6	.34	3.6	82	1.4	98.6 (HPLC)		95.3	4.7
VI-3	M-8	12.3	.46	4.9	91	2.6	0.3	—	—	—
VI-4	M-6	10.9	.42	6.0	67	4.2	0.9	1.8	94.3	5.7

Abbreviations: PCV, packed cell volume; MCV, mean cell volume.

*Hematological data on freshly collected blood samples; the propositus was splenectomized, which increased his Hb level from 8 to 9 g/dL to above 10 g/dL.

†By alkali denaturation.[3]

‡By a chemical procedure.[4]

§By reverse HPLC.[7]

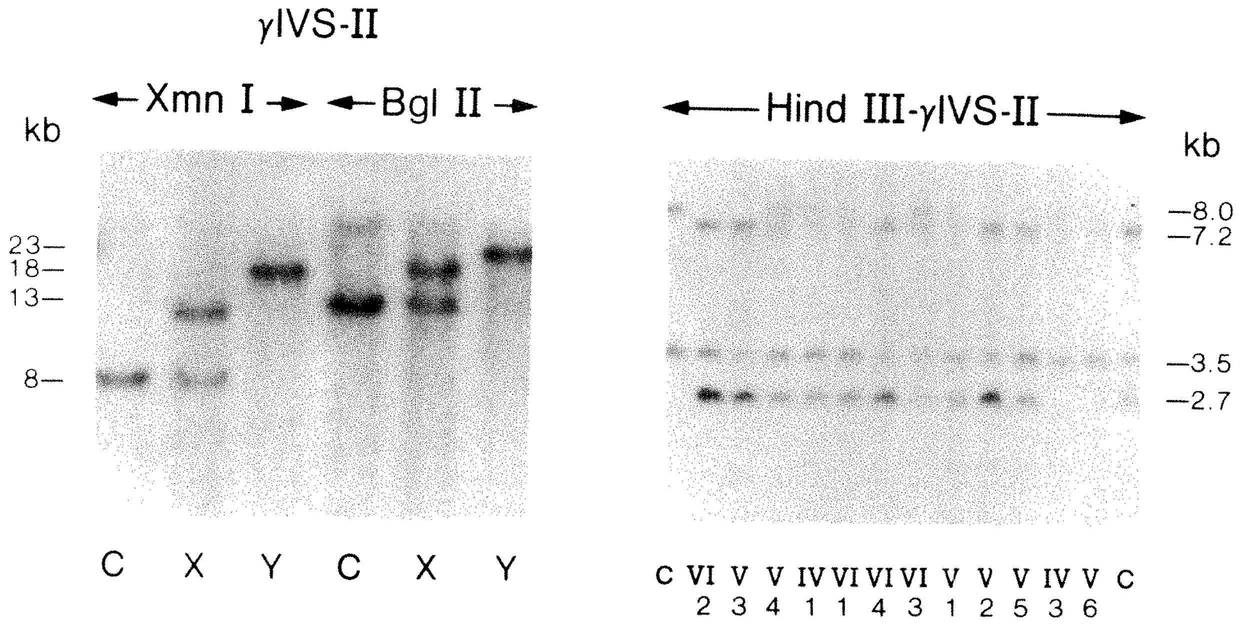

Fig 2. Photographs of X-ray films illustrating restriction fragments in DNA samples from the propositus, from several of his relatives, and from a few controls. (A) c, normal control; x, baby with γ globin gene triplication on one chromosome; Y, propositus with γ globin gene quadruplication on both chromosomes. (B) The samples correspond to the subjects listed in Table 1; two controls (c) are included. Densitometric scanning gave ratios between 2.7-kb and 3.5 kb fragments of 2.13 (patient VI-2); of 2.96, 2.95, and 2.85 (samples V-3, IV-4, and V-2); and of 0.93, 0.88, 0.98, 1.00, 0.92, and 0.89 (samples V-4, IV-1, VI-1, VI-3, V-1, and V-5). Subjects IV-3 and V-6, both normal, had the 3.5-kb fragment only.

which resulted in a $^{G}\gamma$-type promoter region 5′ to the second $^{G}\gamma$-globin gene causing high $^{G}\gamma$ production, while in the Turkish patient this crossover was between bases 6905 and 7085, resulting in an $^{A}\gamma$-type promoter region 5′ to the second $^{G}\gamma$-globin gene and in a lower $^{G}\gamma$ production. Perhaps the quadruplication in our patient is of the -$^{G}\gamma$-$^{G}\gamma$-$^{G}\gamma$-$^{A}\gamma$-type, since the *Hind*III polymorphic site, known to occur frequently in $^{G}\gamma$- but not in $^{A}\gamma$-globin genes,[18-20] was observed in three of the four γ-globin genes (Fig 2). Thus, one can speculate that this quadruplication was the result of a second crossover between a chromosome with a -$^{G}\gamma$-$^{G}\gamma$-$^{A}\gamma$-triplication and a normal -$^{G}\gamma$-$^{A}\gamma$-chromosome. Sequence analyses will be required to further evaluate this possibility. It is interesting that the contribution of the two $^{A}\gamma$-globin genes to the total γ chain production is only 5%.

The consanguinity in this family has limited the number of haplotypes of the normal chromosomes. The three types listed in Fig 1 and Table 2 occurred in the three normal members as well as in the eight β-thalassemic heterozygotes. Table 2 compares the HbF data for these eight adults. The four β-thalassemia traits with the normal haplotype (+ − − − − − + + +] and the one β-thalassemia trait with the normal haplotype [− − + − + + + + −] had significantly lower $^{G}\gamma$ values (85.0% to 88.0%) than the three β-thalassemia traits with the normal haplotype [− − + + − + + + +] with $^{G}\gamma$ values of about 95%. No difference in total γ chain production was evident. A recent study of normal female adults with a homozygosity for haplotype [+ − − − − − + + +] showed low HbF levels with extremely low $^{G}\gamma$ values of about 10%.[21] Thus, the contribution by the normal chromosome with this haplotype (labeled type C in ref 21) and perhaps also by that with haplotype [− − + − + + + + −] (Table 2) to the γ chain composition of the HbF of these β-thalassemia heterozygotes concerns primarily the $^{A}\gamma$ chain, thus lowering the total $^{G}\gamma$ level. This incidental observation confirms our previous data,[21] suggesting that chromosomes with specific haplotypes are associated with distinct $^{G}\gamma$-to-$^{A}\gamma$ ratios in the HbF that is produced.

NOTE ADDED IN PROOF

While this paper was under consideration for publication, Dr J.Z. Liu succeeded in showing that the β^{0}-thalassemia in this family was the result of the deletion of bases 74 and 75 in the codon for amino acid residue number 8 of the β chain. The deletion of this AA dinucleotide was first observed by S.H. Orkin and S.C. Goff (J Biol Chem 256:9782, 1981) in other β-thalassemia patients from Turkey.

Table 2. HbF and G_{γ} Percentages in A-(γγγγ)-β°-Thalassemia Heterozygotes With Normal Chromosomes With Specific Haplotypes

Case	Haplotype of Normal Chromosome	F_{Ile} %	$^{G}\gamma$ %
IV-1	+ − − − − − + + +	0.9	88.0
V-1	+ − − − − − + + +	4.2	87.2
V-4	+ − − − − − + + +	0.8	87.0
VI-1	+ − − − − − + + +	0.5	85.0
V-2	− − + + − + + + +	4.2	95.1
V-3	− − + + − + + + +	1.4	95.3
VI-4	− − + + − + + + +	0.8	94.3
V-5	− − + − + + + + −	1.8	87.9

Our data were obtained as follows: A 10.5-kb *Bcl*I fragment containing the β gene was cloned with EMBL 3 phage. A 2.9-kb fragment containing the β gene was prepared and purified from an *Hpa*I/*Pst*I double digest of the recombinant EMB-β° DNA. Fragments of an *Rsa*I/*Hae*III double digest of this 2.9-kb fragment were subcloned into phage M13mP11; one subclone contained the DNA insert which originated 5′ to the β globin gene and contained the exon 1 (bases from −76 to +132). Sequencing by the dideoxy method identified a 5′ TGAGGAG—GTCTG 3′ sequence replacing the normal 5′ TGAGGAGAAGTCTG 3′ sequence.

REFERENCES

1. Huisman THJ, Jonxis JHP: The Hemoglobinopathies Techniques of Identification. New York, Dekker, 1977
2. Schleider CTH, Mayson SM, Huisman THJ: Further modification of the microchromatographic determination of hemoglobin A_2. Hemoglobin 1:503, 1977
3. Betke K, Marti HR, Schlicht I: Estimation of small percentages of foetal haemoglobin. Nature 184:1877, 1959
4. Schroeder WA, Huisman THJ, Shelton JR, Wilson JB: An improved method for quantitative determination of human fetal hemoglobin. Anal Biochem 35:235, 1970
5. Wilson JB, Headlee ME, Huisman THJ: A new high performance liquid chromatographic procedure for the separation and quantitation of various hemoglobin variants in adults and newborn babies. J Lab Clin Med 102:174, 1983
6. Schroeder WA, Huisman THJ: The Chromatography of Hemoglobin. New York, Dekker, 1980
7. Shelton JB, Shelton JR, Schroeder WA: High performance liquid chromatographic separation of globin chains on a large-pore C_4 column. J Liq Chromatogr 7:1969, 1984
8. Poncz M, Solowiejczyk D, Harpel B, Mory Y, Schwartz E, Surrey S. Construction of human gene libraries from small amounts of peripheral blood: Analysis of β-like globin genes. Hemoglobin 6:27, 1982
9. Kutlar A, Gardiner MB, Headlee MG, Reese AL, Cleek MP, Nagle S, Sukumaran PK, Huisman THJ: Heterogeneity in the molecular basis of three types of hereditary persistence of fetal hemoglobin and the relative synthesis of the $^{G}\gamma$ and $^{A}\gamma$ types of γ chain. Biochem Genet 22:21, 1984
10. Sukumaran PK, Nakatsuji T, Gardiner MB, Reese AL, Gilman JG, Huisman THJ: Gamma thalassemia resulting from a deletion of a γ-globin gene. Nucleic Acids Res 11:4635, 1983
11. Gilman JG, Huisman THJ, Abels J: Dutch β°-thalassaemia: a 10 kilobase DNA deletion associated with a significant γ-chain production. Br J Haematol 56:339, 1984
12. Felice AE, Cleek MP, McKie K, McKie V, Huisman THJ: The rare α-thalassemia-1 of Blacks is a $\zeta\alpha$-thalassemia-1 associated with deletion of all α and ζ globin genes. Blood 63:1253, 1984
13. Felice AE, Cleek MP, Marino EM, McKie K, McKie V, Chang BK, Huisman THJ: Different ζ globin genes among black Americans. Hum Genet 73:221, 1986
14. Harano K, Harano T, Kutlar F, Huisman THJ: γ-Globin gene triplication and quadruplication in Japanese newborns. FEBS Lett 190:45, 1985
15. Shimasaki S, Iuchi I: Diversity of human γ-globin gene loci including a quadruplicated arrangement. Blood 67:784, 1986
16. Liu JZ, Cao Q, Huisman THJ: Replacement of the 5′ promotor region may alter the in vivo activity of a human γ globin gene. (manuscript in preparation)
17. Efremov GD, Filipce V, Gjorgovski I, Juricic D, Stojanovski N, Harano T, Nakatsuji T, Kutlar A, Kutlar F, Bakioglu I, Huisman THJ: $^{G}\gamma^{A}\gamma(\delta\beta)$ °-Thalassaemia and a new form of γ globin gene triplication identified in the Yugoslavian population. Br J Haematol 63:17, 1986
18. Orkin SH, Kazazian HH, Jr, Antonarakis SE, Goff SC, Boehm CD, Sexton JP, Waber PG, Giardina PJV: Linkage of β-thalassaemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. Nature 296:627, 1982
19. Orkin SH, Kazazian HH Jr: The mutation and polymorphism of the human β-globin gene and its surrounding DNA. Annu Rev Genet 18:131, 1984
20. Shen SH, Slightom JL, Smithies O: A history of the human fetal globin gene duplication. Cell 26:203, 1981
21. Hattori Y, Kutlar F, Mosley CJ, Mayson SM, Huisman THJ: Association of the level of $^{G}\gamma$ chain in the fetal hemoglobin of normal adults with specific haplotypes. Hemoglobin 10:185, 1986

CONCISE REPORT

Substance P Receptors in Rat Spleen: Characterization and Autoradiographic Distribution

By Christian J. Wiedermann, Kaspar Sertl, and Candace B. Pert

The interaction of substance P with intact lymphatic tissue was quantified and autoradiographically visualized, using slide-mounted tissue sections of rat spleen. Radiolabeled substance P binds rapidly to an apparently single class of noninteracting high affinity sites ($K_d = 2.4$ nmol/L; $B_{max} = 9.4$ fmol/mg protein). The ligand selectivity pattern suggests that substance P binding sites are similar to substance P receptors found in other tissues, including the brain, T lymphocytes, and macrophages. Substance P receptors are highly concentrated in the antigen-trapping spleen marginal zone, with low densities being found in the red pulp. No specific binding of radiolabel to T cell-dependent immunologic domains of the spleen is seen. The distribution of substance P receptors suggests that substance P is probably involved in the control of sensory functions of the immune system.

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SUBSTANCE P (SP), an undecapeptide of amino acid sequence, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, is found to be present in the central and peripheral nervous system and in gut.[1,2] Preliminary results also suggest the presence in a bone marrow–originating cell type.[3] In the central nervous system, SP is a putative pain neurotransmitter,[1] and in the intestinal tract it evokes potent excitatory actions.[1] In sensory nerves supplying local sites of chronic inflammation, elevated levels of SP have been detected, thus implicating a role for SP to play in mediation of allergic reactions.[4] Upon antidromic release from these primary afferents, SP was found to cause an inflammatory response,[5] and it is demonstrated that SP modulates the severity of joint injury in experimental arthritis.[6] Subsequently SP has been shown to affect functions of mast cells,[3] polymorphonuclear leukocytes,[7] monocytes/macrophages,[8,9] and lymphocytes.[10,11] These studies suggest the existence of specific, functional receptors for SP on different types of blood cells. Direct labeling of SP receptors was achieved on cultured human IM-9 lymphoblasts,[12] on subpopulations of human T and B lymphocytes,[13,14] and on guinea pig peritoneal macrophages.[15] Since SP receptor expression does not seem to be a characteristic of immune cells *per se*, we studied the presence of SP receptors in lymphatic tissue, and we report the autoradiographic localization of in vitro ^{125}I-labeled SP receptors in rat spleen.

MATERIALS AND METHODS

Preparation of sections. As described previously,[16] 24-μm-thick serial sections of frozen rat (adult female, Sprague-Dawley) spleens were thaw-mounted onto subbed slides, placed under vacuum in a chilled desiccator jar, stored overnight between 0 °C and 4 °C and assayed the next day. Protein determinations[17] of representative samples of sections were performed.

Assay. Slides with two spleen sections mounted on each were placed in stainless steel racks and immersed in a medium of 158 mmol/L NaCl, 4.75 mmol/L KCl, 1.27 mmol/L $CaCl_2$, 1.42 mmol/L $MgCl_2$, 11 mmol/L dextrose, 15 mmol/L Tris-HCl at pH 7.4, containing 0.2 mmol/L guanosine 5′-triphosphate (Sigma, St. Louis). Following a 30-minute incubation, sections were washed by two sequential five-minute incubations in 500 mL of 50 mmol/L Tris-HCl buffer, pH 7.4. To label SP receptors, incubations were performed in an assay medium consisting of 50 mmol/L Tris-HCl buffer, pH 7.4, 50 μg/mL bacitracin, 4 μg/mL chymostatin, 4 μg/mL leupeptin, 5 mg/mL bovine serum albumin (all from Sigma, St. Louis), 3 mmol/L $MgCl_2$, and 50 nmol/L iodinated Bolton-Hunter–coupled SP ([^{125}I]BH-SP; New England Nuclear, Pittsburgh). Following incubation at room temperature, the slides were washed by four sequential two-minute incubations in ice-cold buffer containing 3 mmol/L $MgCl_2$. They were dried under a stream of cold air. Nonspecific binding was defined as binding in the presence of 1 μmol/L SP, and it was normally below 15% of total binding.

For biochemical experiments, individual sections were scraped off slides and bound radioactivity was determined by gamma counting (efficiency 0.79%). For autoradiography, the sections were fixed with hot formaldehyde vapor[18]; slides were placed in X-ray cassettes and exposed to tritium-sensitive film ([^{3}H]Ultrofilm; LKB, Rockville, MD). Films were developed after five days of exposure. Slides were then defatted and dipped in liquid emulsion (NTB-2; Kodak, Rochester, NY), exposed for 14 days at 4 °C, developed, and counterstained with hematoxylin-eosin.

Peptides were purchased from Peninsula Laboratories (Belmont, CA).

RESULTS

Characterization of [^{125}I]BH-SP binding to slide-mounted sections. The time course of association of [^{125}I]BH-SP was assessed in two separate experiments by incubation of duplicate slides (two spleen sections mounted on each) with 0.1 nmol/L [^{125}I]BH-SP for 3 to 120 minutes. At room temperature, binding reached a plateau within approximately 45

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minutes, being half-maximal at 12 minutes with a pseudo-first order rate constant K_{obs} of 0.055 min^{-1} (Fig 1A). Incubation time of further experiments was 40 minutes.

Figure 1B illustrates that specific binding appeared to be saturable. The lower inset of Fig 1B shows that a double-reciprocal plot of the specific binding data yields a straight line ($r = 0.95$), suggesting that binding occurs to a single class of sites. From this plot a K_d of 2.4 nmol/L and a B_{max} of 9.4 fmol/mg protein were calculated. [^{125}I]BH-SP appeared to bind to noninteracting sites because a Hill plot of the specific binding data (Fig 1, upper inset) yielded a line with a slope of 0.93 ($r = 0.94$).

SP is highly potent in binding competition experiments against [^{125}I]BH-SP, and SP[3-11] possesses appreciable activity (Fig 2). Mean concentrations ($n = 3$) displacing 50% of specifically bound [^{125}I]BH-SP (IC_{50}) were 0.55 × 10^{-8} mol/L and 0.80 × 10^{-6} mol/L, respectively. In contrast, similar concentrations of SP[1-4] failed to significantly inhibit the binding.

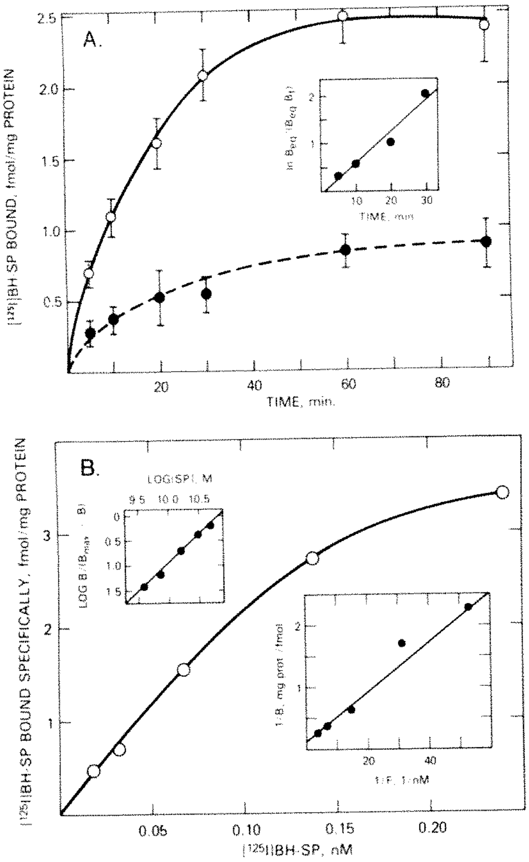

Fig 1. (A) Kinetics of [^{125}I]BH-SP association to rat spleen incubated with 0.1 nmol/L of radiolabel at 22 °C for indicated periods of time. Points are the means ± standard deviations of two experiments each performed in two duplicates. Inset: Pseudo-first order rate plot of the data. B, specific binding; eq, at equilibrium; t, at incubation time. (B) Binding of [^{125}I]BH-SP as a function of radiolabeled peptide concentration. Points are the means of triplicate assays; results are representative of two experiments. Upper inset: Hill plot of the data by using the B_{max} of 9.4 fmol/mg protein obtained in the double-reciprocal plot. Lower Inset: Double-reciprocal plot of the data F = [free ligand]; B = [bound ligand].

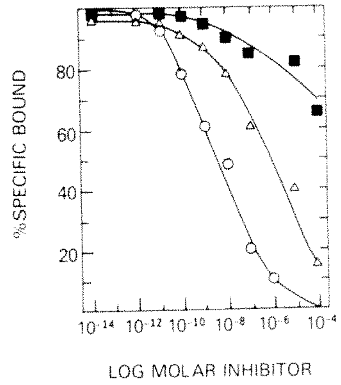

Fig 2. Pharmacology of SP binding to rat spleen. Specific binding of 0.1 nmol/L [^{125}I]BH-SP as a function of unlabeled SP (○), SP[3-11] (△) and SP[1-4](■). The mean value is indicated for three separate experiments each conducted in two duplicates.

Autoradiography. The regional distribution of SP receptors within the spleen was investigated using in vitro autoradiographic techniques. SP receptors are concentrated in the red pulp (Fig 3A). Highest grain densities are found in the marginal zone (Figs 3A, 3C, and 3D). Controls treated with 10^{-6} mol/L unlabeled SP show minimal binding (Figs 3B, 3E, and 3F). No specific binding to structures of periarteriolar lymphoid sheath or peripheral white pulp can be seen.

DISCUSSION

The present biochemical results clearly indicate the presence of SP receptors in rat spleen. [^{125}I]BH-SP bound with high affinity to a single class of noninteracting receptors (Fig 1). The affinity is compatible with that of [^{125}I]BH-SP binding to SP receptors in rat brain slices[19] and on synaptosomal membranes.[20] Specificity of this high-affinity binding has been confirmed by obtaining the appropriate relative potencies of SP, SP[3-11], and SP[1-4] in competing with radiolabeled SP (Fig 3). As described for SP receptors in rat brain,[21] SP[3-11] is about 70 times less effective in [^{125}I]BH-SP binding inhibition than SP. The importance of the carboxyterminal amino acids of SP for binding is indicated by the relative weakness of SP[1-4] in displacing radiolabel. The data are in agreement with results obtained for human circulating T lymphocytes,[13] and guinea pig peritoneal macrophages.[15]

Autoradiographic visualization of [^{125}I]BH-SP binding sites shows that SP receptors are distinctively distributed (Fig 3). Specific grains are present in red pulp. Highest grain densities are located to the marginal zone (MZ). Surprisingly, no specific binding was visible in T lymphocytic domains of the spleen, ie periarteriolar lymphoid sheath,[22] even though 7,000 receptors per cell were demonstrated on subpopulations of human peripheral blood T lymphocytes.[13] Direct and/or indirect evidence has been obtained for the presence of SP receptors on several types of immune cells, including T lymphocytes, B lymphocytes, polymorphonuclear leukocytes, monocytes, and macrophages. From this we conclude that the functional significance of SP receptors in the immune system depends on the physiological context.

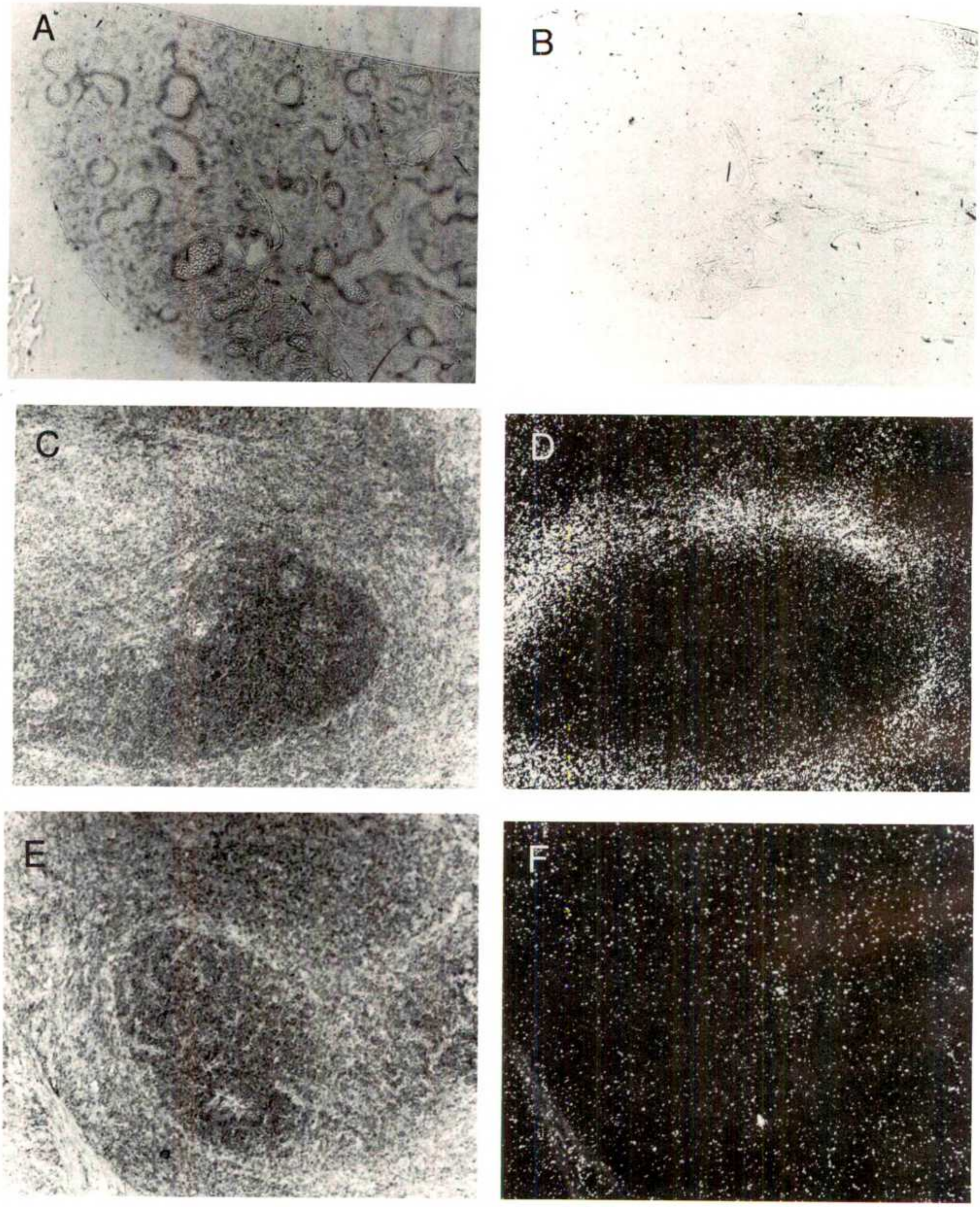

Fig 3. Autoradiographs of [^{125}I]BH-SP receptor distribution in rat spleen. Slide-mounted sections were incubated at optimum conditions in 0.1 nmol/L [^{125}I]BH-SP in the absence (A, C, D) or presence (B, E, F) of 1 μmol/L unlabeled SP. (A) Distribution of grains on fixed sections without counterstain; magnification 5×. (B) Control for (A). (C) Higher magnification (×400), counterstained with hematoxylin-eosin and (D) corresponding darkfield photomicrograph. (E, F) Control for (C, D).

The MZ at the border of white pulp and red pulp of the spleen is a unique structure. Terminal branches of the arterial system in the white pulp discharge their contents between the cells of the MZ, in order for the cells to meet antigens arriving in the spleen.[22] The antigen is picked up from the MZ by a subpopulation of red pulp mononuclear phagocytes, so-called "marginal metallophyls," and transported into the white pulp to initiate specific immune responses.[23] Thus, the presence in high concentration of SP receptors in the MZ indicates potential importance of SP in mediation of the immune systems afferent limb function; enhancing effects of SP on phagocytosis,[7] cell migration,[9] and antibody production[11] have been reported in vitro. Utilizing autoradiography, we are therefore able to correlate SP receptor expression with a functional immunologic domain in intact lymphatic tissue.

Based on the data presented in this report, no direct conclusion for the cell type that is bearing SP receptors can be drawn. Indirect evidence suggests that specialized splenic macrophages/marginal metallophyls might display SP receptors: (1) distribution patterns for SP receptors (Fig 3) and marginal metallophyls which are typically and most abundantly present in MZ[22] appear to be identical, and (2) other cell types that are normally found in MZ, ie reticular fibrocytes, condensed sinus lining cells, thrombocytes, granulocytes, and T and B lymphocytes, are found in other spleen areas as well[23] where no visible [^{125}I]BH-SP labeling occurred. Previously, the presence in the MZ of a subpopulation of nonrecirculating B cells has been proposed[24] that theoretically might expose SP receptors, but in recent studies no special relationship between antibody forming cells and MZ macrophages was found.[25]

In conclusion, the present results and those of an earlier study on receptors for vasoactive intestinal peptide[16] show that neuropeptides possess recognition molecules in spleen which are distinctively distributed. These findings may provide a biochemical basis for the increasingly realized selective modulation of immune functions by neuropeptides.[26,27]

REFERENCES

1. Ersparmer V: The tachykinin peptide family. Trends Neurosci 4:267, 1981
2. Leeman SE, Gamse R: Substance P in sensory neurons. Trends Pharmacol Sci 2:119, 1981
3. Goetzl EJ, Chernov-Rogan T, Furuichi K, Goetzl LM, Lee JY, Renold F: Neuromodulation of mast cell and basophil function, in Befus AD, Bienenstock J, Denburg JD (eds): Mast Cell Differentiation and Heterogeneity. New York, Raven, in press
4. Lembeck F, Donnerer J, Colpaert FC: Increase of substance P in primary afferent nerves during chronic pain. Neuropeptides 1:175, 1981
5. Lembeck F, Holzer P: Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. Naunyn-Schmiedeberg's Arch Pharmacol 310:175, 1979
6. Levine JD, Clark R, Helms C, Moskowitz MA, Basbaum AI: Intra-neuronal substance P contributes to the severity of experimental arthritis. Science 226:547, 1984
7. Bar-Shavit Z, Goldman R, Stabinsky Y, Gottlieb B, Fridkin M, Teichburg VI, Blumberg S: Enhancement of phagocytes—A newly found activity of substance P residing in its N-terminal tetrapeptide sequence. Biochem Biophys Res Comm 94:1445, 1980
8. Hartung HP, Toyka KV: Activation of macrophages by substance P: Induction of oxidative burst and thromboxane release. Eur J Pharmacol 89:301, 1983
9. Ruff MR, Wahl SM, Pert CB: Substance P receptor-mediated chemotaxis of human monocytes. Peptides 6:107, 1985 (suppl 2)
10. Payan DG, Brester DR, Goetzl EJ: Specific stimulation of human T lymphocytes by substance P. J Immunol 131:1613, 1983
11. Stanisz AM, Befus D, Bienenstock J: Differential effects of vasoactive intestinal peptide, substance P, and somatostatin on immunoglobulin synthesis and proliferation of lymphocytes from Peyers' patches, mesenteric lymph nodes, and spleen. J Immunol 133:152, 1986
12. Payan DG, Brewster DR, Goetzl EJ: Stereospecific receptors for substance P on cultured human IM-9 lymphocytes. J Immunol 133:3260, 1984
13. Payan DG, Brewster DR, Missirian-Bastain A, Goetzl EJ: Substance P recognition by a subset of human T lymphocytes. J Clin Invest 74:1532, 1984
14. Stanisz AM, Scicchitano R, Payan DG, Bienenstock J: In vitro studies of immunoregulation by substance P and somatostatin. Second International Workshop on Neuroimmunomodulation. Dubrovnik, June 1986
15. Hartung HP, Walters K, Toyka KV: Substance P: Binding properties and studies on cellular responses in guinea pig macrophages. J Immunol 136:3856, 1986
16. Wiedermann CJ, Sertl K, Zipser B, Hill JM, Pert CB: Vasoactive intestinal peptide receptors in rat spleen and brain: A shared communication network. Peptides (in press)
17. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 193:265, 1951
18. Herkenham M, Pert CB: Light microscopic localization of brain opiate receptors: A general autoradiographic method which preserves tissue quality. J Neurosci 2:1129, 1982
19. Rothman RB, Herkenham M, Pert CB, Liang T, Cascieri MA: Visualization of rat brain receptors for the neuropeptide, substance P. Brain Res 309:47, 1984
20. Viger A, Benjouan JC, Torreus Y, Glowinsky Y: Specific binding of a ^{125}I-substance P derivative to rat brain synaptosomes. J Neurochem 40:1030, 1983
21. Quirion R, Shults CW, Moody TW, Pert CB, Chase TN, O'Donohue TL: Autoradiographic distribution of substance P receptors in rat central nervous system. Nature 303:714, 1983
22. Eikelenboom P, Dijkstra CD, Boorsma DM, van Rooijen N: Characterization of lymphoid and nonlymphoid cells in the white pulp of the spleen using immunoperoxidase techniques and enzymehistochemistry. Experientia 41:209, 1985
23. van Ewijk W, Nieuwenhuis P: Compartments, domains and migration pathways of lymphoid cells in the splenic pulp. Experientia 41:199, 1985
24. MacLennan ICM, Gray D, Kumararatne DS, Bazin H: The lymphocytes of splenic marginal zones: A distinct B cell lineage. Immunol Today 3:305, 1982
25. Claassen E, Kors N, Dijkstra CD, van Rooijen N: Marginal zone of the spleen and the development and localization of specific antibody-forming cells against thymus-dependent and thymus-independent type-2 antigens. Immunology 57:399, 1986
26. Goetzl EJ (ed): Neuromodulation of immunity and hypersensitivity, Proceedings of a Conference. J Immunol 135:1s, 1985
27. Pert CB, Ruff MR, Weber RJ, Herkenham M: Neuropeptides and their receptors: A psychosomatic network. J Immunol 135:821s, 1985

CONCISE REPORT

Hyposialylation of Differentiation-Inducer–Resistant HL-60 Cells

By Robert E. Gallagher, Dennis A. Giangiulio, Chin-Sen Chang, Constance J. Glover, and Ronald L. Felsted

The total sialic acid concent of retinoic acid (RA)-resistant or 6-thioguanine (6TG)-resistant HL-60 cells was more than tenfold lower and of dimethylsulfoxide (DMSO)-resistant HL-60 cells was approximately twofold lower than that of parental, wild-type (wt) HL-60 cells. Neuraminidase-inaccessible, ie residual cell-associated sialic acid after neuraminidase treatment, was four- to twelvefold lower in the three differentiation-inducer–resistant sublines than in the parent line. Neuraminidase treatment of ^{125}I-labeled surface membrane glycoproteins (SMGs) from wt HL-60 cells converted the two-dimensional gel electrophoretic pattern to one having features in common with RA- and 6TG-resistant cells. However, neuraminidase treatment did not alter the sensitivity of wt HL-60 cells to differentiation induction by RA, hypoxanthine (purine base), or DMSO. These results indicate that differences in peripheral, neuraminidase-accessible sialic acids are important determinants of the gel electrophoretic mobility of the SMGs of the HL-60 line and sublines but are not likely related to the differentiation-resistance mechanism. Further studies are required to determine if hyposialylation of cryptic, neuraminidase-inaccessible sites has functional significance.

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VARIATIONS IN surface membrane glycoprotein (SMG) sialylation have been associated with a variety of important altered cellular states.[1] Although results have been variable in different biologic systems, increased sialylation of surface membrane oligosaccharides of malignant cells relative to normal cell controls, including chronic myelogenous leukemia cells *v* normal granulocytes, has most frequently been reported.[2,3] These alterations have been related to changes in cell-cell interaction, adherence, and metastatic potential. Changes in sialylation have also been noted to occur as a function of cellular differentiation, including response to differentiation induction by retinoic acid (RA) and dimethylsulfoxide (DMSO).[4,5] Although such studies have not, to our knowledge, been reported using HL-60 cells, variations in sialytransferase activity[6,7] and in sialidase (neuraminidase) activity[8] have been reported as a function of HL-60 cell granulocytic differentiation. Finally, with respect to drug resistance, increased sialylation was described in one study of Actinomycin D–resistant hamster cells,[9] while a preliminary report indicated that surface hyposialylation occurs in Adriamycin-resistant HL-60 cells.[10]

The present study was based on the use of three sublines of HL-60 cells which were selected for their ability to continue logarithmic growth in the presence of either RA, 6-thioguanine (6TG) or DMSO.[11,12] These sublines are variably resistant/cross-resistant to the granulocytic differentiation–inducing effects of these three selective agents but are not cross-resistant to inducers of monocyte/macrophage differentiation.[12] The RA-resistant and 6TG-resistant sublines were discovered to have SMG gel electrophoretic patterns which markedly differed from those of wild-type (wt) and DMSO-resistant HL-60 cells but which were virtually identical to one another.[13] The present study was formulated to determine if the changes noted in the variant SMG gel electrophoretic profile could be related to sialylation differences, since they involved possible shifts to more basic positions of wt SMG species,[13] and, if so, to determine if experimentally altering sialylation by neuraminidase treatment has an effect on the sensitivity to granulocytic differentiation inducers.

MATERIALS AND METHODS

Cell culture lines and methods. The RA-resistant, 6TG-resistant and DMSO-resistant HL-60 sublines were derived and maintained as previously described.[11,12] Differentiation induction assays with 10^{-6} mol/L RA for five days, and 169 mmol/L DMSO or 6 mmol/L hypoxanthine for seven days, including the monitoring of granulocytic differentiation by cytologic examination of Wright stained smears and by the nitroblue tetrazolium (NBT) dye reduction test, were as previously described.[11,12] Short-term neuraminidase treatment (with *Vibrio cholerae*, Berhringwerke Marburg, FRG) of wt HL-60 cells prior to differentiation induction experiments (pretreatment) was performed using phosphate buffered saline (PBS)-washed cells, as described below. Continuous neuraminidase treatment involved adding 0.05 IU/mL neuraminidase to the complete tissue culture medium immediately after reestablishment in culture following neuraminidase pretreatment and replenishing with medium containing fresh neuraminidase on days 3 and 7. All biochemical procedures were performed on cells harvested from the mid- to late-log phase of growth, when cell viability exceeded 90% by trypan blue dye exclusion.

Determination of total and neuraminidase-releasable sialic acid. Cell suspensions of approximately 10^8 wt or variant HL-60 cells were washed three times in 5 mL PBS with 1 mg Ca^{++} per liter (PBS-Ca^{++}, GIBCO, Grand Island, NY). For determination of total sialic acid, the washed cells were suspended in 0.5 mL of 0.1N H_2SO_4 at 80 °C for one hour to hydrolyse glycosidically-linked sialic acids and release sialic acid from intracellular sites,[14] followed by the removal of cell debris by centrifugation at 1,000 × g for five minutes. For determination of neuraminidase-releasable sialic acid, cells were suspended in 0.5 mL of PBS-Ca^{++}, pH 7.4, containing 0.05 IU

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Vibrio cholerae neuraminidase (0.1 IU/mL) and incubated at 37 °C for 15 minutes, followed by clarification by centrifugation at 200 × g for seven minutes. Kinetic experiments demonstrated that under these conditions neuraminidase was present in considerable excess, since with the use of 0.02 IU/mL the release of sialic acid was complete in 15 minutes. The sialic acid content of the hydrolysates was directly determined by the thiobarbituric acid colorimetric assay,[15] as modified by Roboz et al to eliminate 2-deoxyribose interference,[16] using sialic acid from sheep submaxillary glands (Sigma, St Louis) as a standard. Digests of 2 × 10^7 cells were assayed for protein content according to the method of Bradford,[17] using bovine serum albumin as a protein standard. Each sialic acid measurement represents the average of three independent tests. The neuraminidase-releasable sialic acid was calculated as the difference between that measured in the presence and absence of the enzyme. The statistical significance of the variations of the sialic acid determinations in the variant *v* wt HL-60 cells was calculated by Students *t* test.

SMG gel electrophoretic procedures. Two × 10^6 viable cells were surface-labeled with I-125 by the Iodo-Gen (Pierce Chemical, Rockford, IL) method using 0.5 mCi sodium-^{125}I (New England Nuclear, Boston) as previously detailed.[13,18] The labeled and washed cells were treated with diisopropylfluorophosphate and extracted with ice-cold 2% Triton X-100 (Sigma) in PBS with Ca^{2+} containing 2 mmol/L phenylmethylsulfonylfluoride (Sigma).[18] Neuraminidase treatment was performed on the labeled, washed cells (Fig 1) or on

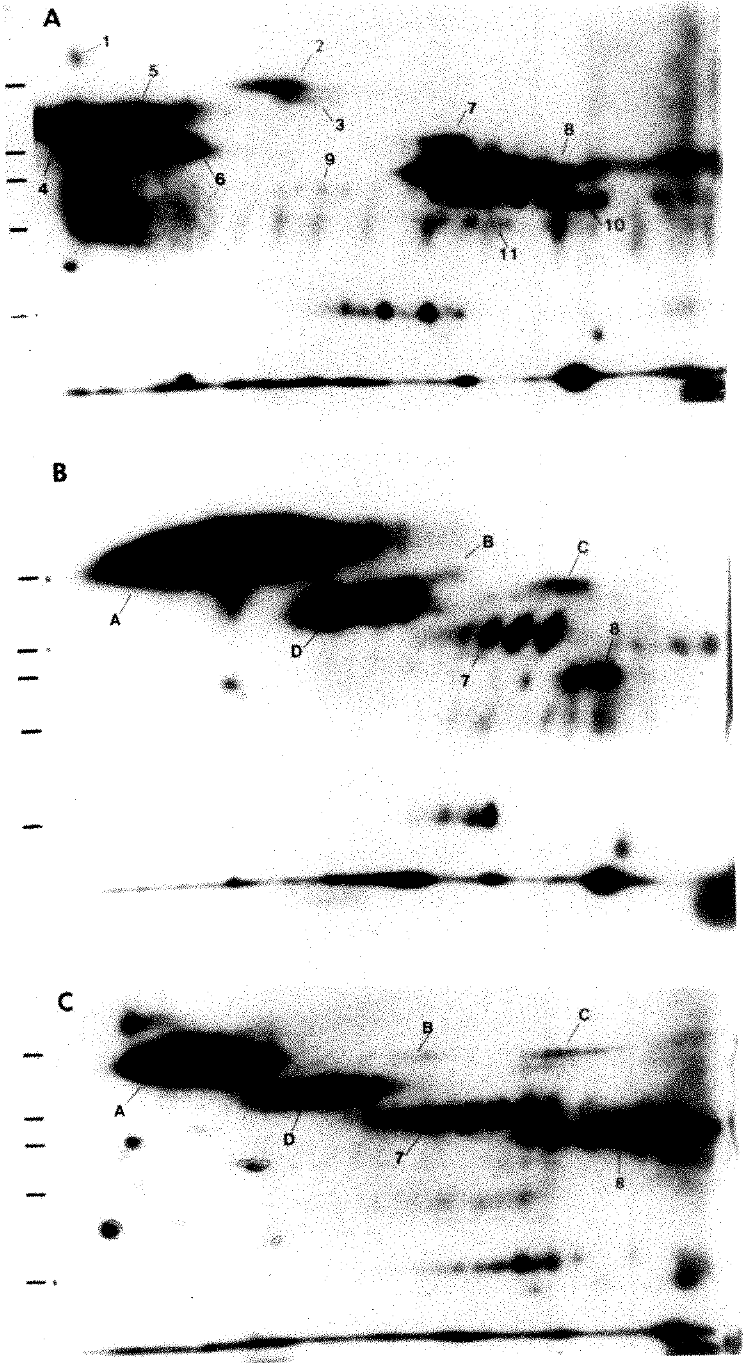

Fig 1. Autoradiographs of surface proteins after I-125 labeling and two-dimensional polyacrylamide gel electrophoresis. The first (horizontal) dimension was isoelectric focusing with an acidic pH of 4.5 on the extreme left and an alkaline pH of 8.0 on the extreme right. The second (vertical) dimension was SDS-polyacrylamide gel electrophoresis from top to bottom in which the indicated molecular weight markers correspond to 200, 116, 93, 69, and 45 kD. The nomenclature was derived from graphic displays of SMG spots from multiple radioautographic exposures as described in ref 13. Cell sources: (A), wt HL-60; (B), RA-resistant HL-60; (C), neuraminidase-treated wt HL-60.

the Triton X-100 protein extracts at 0.1 IU/mL under conditions described in the previous section, which produced a similar, complete reaction from five to >30 minutes of treatment. The I-125–labeled extracts were subjected to isoelectric focusing followed by polyacrylamide gel electrophoresis as described previously,[18] calibrating the second dimension with mixed high molecular weight standard proteins (myosin, 200,000; β-galactosidase, 116,000; phosphorylase B, 92,000; bovine serum albumin, 69,000; and ovalbumin, 45,000) (Bio-Rad, Richmond, CA).

RESULTS

Quantitation of total and neuraminidase-releasable sialic acid. Both the RA- and 6TG-resistant sublines had less than 10% of the total amount of sialic acid present in wt HL-60 cells (Table 1). The amount of sialic acid in the DMSO-resistant cells was also significantly lower at approximately 45% of the wt cellular level. Qualitatively similar results were obtained by the periodate-resorcinol method,[19] but we were unable to obtain quantitatively highly reproducible results due to the presence of variable amounts of interfering substances. After neuraminidase treatment, approximately 50% of the sialic acid residues were releasable from the wt HL-60 cells (Table 1). Relatively more sialic acid (73%) was releasable by neuraminidase-treatment of the DMSO-resistant cells. Thus, a four- to twelvefold higher residual amount of neuraminidase-inaccessible sialic acid was present in wt HL-60 cells than in all three variant sublines (Table 1, column 3).

Effect of neuraminidase treatment on SMGs. After ^{125}I-labeling, extraction, and two-dimensional gel electrophoresis, the surface-labeled proteins of HL-60 cells appeared as groups of multiple-charged polypeptides and/or as fused horizontal smears of proteins, as typical of SMGs.[20] From studies of radioautographs after different exposure times, up to 12 distinguishable SMGs could be identified in wt HL-60 cells, while only nine SMGs were distinguishable in RA-resistant cells (ref 13 and Figs 1A and B). The SMG gel electrophoretic pattern of the DMSO-resistant cells closely resembled that of wt cells, while the SMG pattern of the 6TG-resistant cells was very similar to that of the RA-resistant cells.[13] Furthermore, four SMGs present in the RA- or 6TG-resistant cells were not observed in the wt or DMSO-resistant cells (proteins A through D, Fig 1B). Most notably, the wt or DMSO-resistant cells manifested three closely associated, prominent acidic SMGs in the 120- to 160-kD range (proteins 4, 5, and 6, Fig 1A), while the RA-/6TG-resistant cells showed a single predominant acidic SMG of ≥200 kD (protein A, Fig 1B). Treatment of ^{125}I-labeled wild-type cells with *Vibrio cholerae* neuraminidase, converted the SMG gel electrophoretic pattern to one with features resembling that of the RA- or 6TG-resistant cells (Fig 1C). This included the following notable changes: (1) the loss or shift of the three acidic 120- to 160-kD proteins (proteins 4, 5, and 6); (2) the appearance of a prominent higher apparent molecular weight acidic SMG (170- to 120-kD), which resembles protein A but was of significantly less apparent molecular mass; (3) the appearance of a prominent 130- to 150-kD SMG with intermediate isoelectric mobility (protein D); (4) a marked increase in intensity of a 115- to 125-kD, pI 6.0-7.0 SMG (protein 7); and (5) a basic shift of the prominent 85- to 95-kD, pI 6-7 SMG, previously identified as the transferrin receptor in both wt and RA-resistant HL-60 cells (protein 8, Ref 13). Similar results were obtained whether neuraminidase treatment was performed before or after detergent extraction of the SMGs.

Table 1. Total and Neuraminidase-Releasable Sialic Acid Content of Wild-type and Differentiation-Inducer–Resistant HL-60 Cells

	Sialic Acid (μmol/g protein)		
Line/Sub-line	Total (A)	Neuramindase-releasable (B)	Neuraminidase inaccessible (A-B)
Wild-type	19.31 ± 1.21*	9.80 ± 0.50*	9.51
RA-resistant	1.35 ± 0.20†	0.32 ± 0.11†	1.03
6TG-resistant	1.89 ± 0.26†	1.12 ± 0.24†	0.77
DMSO-resistant	8.77 ± 0.62†	6.44 ± 0.32†	2.33

*Values represent the mean of triplicate determinations ± standard deviation.

†Values significantly differ from wild-type determination, $p < 0.01$.

Tests of neuraminidase treatment for effects on growth and differentiation of wt HL-60 cells. The above results raised the possibilities that the hyposialylation and/or altered SMGs of the RA-resistant and 6TG-resistant cells could be mechanistically related to their differentiation resistance and that neuraminidase treatment of the wt HL-60 cells might reduce their sensitivity to differentiation stimulators. We demonstrated by direct sialic acid assay or by the binding of the lectin peanut agglutinin, which binds only to hyposialylated HL-60 cells[21] (and Gallagher et al, manuscript in preparation), that wt HL-60 cells continuously treated with neuraminidase (see MATERIALS and METHODS) were persistently hyposialylated, the lowest recorded level being 6.1 μmol/g protein (compare with neuraminidase-inaccessible values in Table 1). Such treatment did not affect cell growth rate or the overall level of spontaneous differentiation (data not shown). In several dose-response experiments comparing untreated and acutely or continuously neuraminidase-treated cells, no loss of granulocytic differentiation responsiveness to RA (representatively shown in Fig 2), hypoxanthine, or DMSO was detected, as determined by cytologic evaluation of Wright stains or by the NBT test. The cytologic appearance of the induced HL-60 neutrophilic granulocytes was not discernibly different in the untreated and neuraminidase-treated cells.

DISCUSSION

The principle finding of this study is that differentiation-inducer resistant HL-60 cells are hyposialylated compared to wt HL-60 cells. This finding is relevant to issues which we previously raised about these cultured cells.[13] First, it can at least partly account for the differences in SMG gel electrophoretic profiles between the RA-resistant and 6TG-resistant HL-60 cells compared to wt cells, since neuraminidase treatment of wt cells produced a SMG gel profile with features of the variant SMG pattern (Fig 1). In this context,

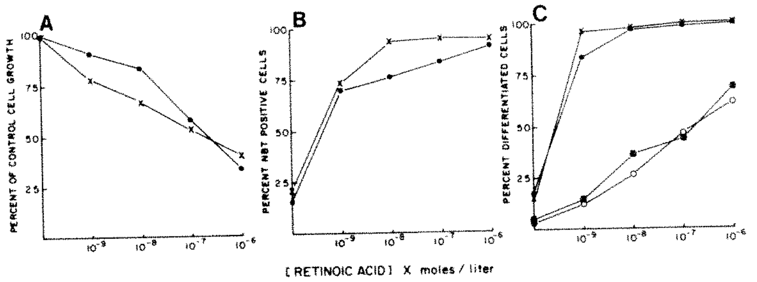

Fig 2. Lack of effect of neuraminidase treatment on the growth and granulocytic differentiation of wt HL-60 cells in the absence and presence of various concentrations of RA. Cells were grown for 4 weeks in the continuous presence of neuraminidase added freshly two times per week to a final concentration of 0.05 IU/mL and at the initiation of the five-day induction period with retinoic acid. (A) Percent of control cell count, ie percent of growth of RA-treated cells to growth of control cells in the absence of RA treatment; (B) percentage of cells positive by the NBT test; (C) percentage of cells at or beyond the myelocytic stage of differentiation. ●, without neuraminidase treatment; X, with neuraminidase treatment. Panel C: ○, percentage mature neutrophils without neuraminidase treatment; ×, percentage of mature neutrophils with neuraminidase treatment.

it is possible to attempt to interpret some of the specific alterations. For example, it seems virtually certain that the basic shift of protein 8 in neuraminidase-treated HL-60 cells (Fig 1) is due to the reduction of negatively-charged sialic acid residues on the N-linked oligosaccharides of this glycoprotein,[22] previously demonstrated to be the transferrin receptor in both wt and RA-resistant HL-60 cells.[13,18] Similarly, protein D (Fig 1) is most likely derived from a basic shift due to hyposialylation of one of the three 120- to 160-kD glycoproteins observed in wt HL-60 cells (protein 4, 5, or 6, Fig 1). On the other hand, the derivation of the prominent acidic SMG protein A (Fig 1) is more difficult to decipher. A not unlikely possibility, however, is that it is also derived from Protein 4, 5, or 6 due to diminished electrophoretic migration of the hyposialylated glycoprotein. Such paradoxically decreased mobility in SDS-polyacrylamide gels of a glycoprotein with reduced molecular mass has been noted to occur after desialylation of densely sialylated O-linked oligosaccharides, eg glycophorin.[23] Proof of the derivation of the variant glycoproteins as well as an understanding of the cause of the hyposialylation will require detailed biochemical analysis.

Second, we previously noted the similarity in the overall SMG gel electrophoretic pattern difference between the RA- or 6TG-resistant cells *v* wt HL-60 cells and those described in pleiotropic, multidrug-resistant cells of various types from different mammalian species.[13] In particular, SMG changes in multidrug-resistant cells have been associated with the reciprocal loss/gain of lower/higher molecular weight SMGs as a function of drug resistance,[24,25] and, in some instances, this has been associated with a marked augmentation of a specific higher molecular weight SMG called the P glycoprotein.[24] The present studies provide circumstantial evidence that the prominent acidic, high-molecular-weight SMG observed in the RA- or 6TG-resistant HL-60 cells is not due to increase of a P glycoprotein-like SMG but rather is due to a shift in a preexistent wt glycoprotein due to hyposialylation effects. Further, using a specific antibody to the P-glycoprotein,[24] no evidence of increased expression of P-glycoprotein was found in sucrose density gradient–purified membranes from the differentiation-inducer–resistant HL-60 cells (unpublished data, September 1985, with J. Gerlach and V. Ling, Toronto).

Finally, the present results suggest that neither the striking SMG differences as detected by two-dimensional gel electrophoresis nor the differences in terminal, neuraminidase-accessible oligosaccharide sialylation are mechanistically related to the resistance/cross-resistance to the granulocytic differentiation inducers in our variant HL-60 cells. These results do not exclude the possibilities that the hyposialylation defect which arose under the tissue culture selection conditions leading to differentiation-inducer–resistance could be a closely associated marker for the resistance phenotype or that the four- to twelvefold deficiency of neuraminidase-inaccessible sialic acid in the differentiation-inducer–resistant HL-60 cells *v* wt HL-60 cells (Table 1) could be functionally related to the resistance/cross-resistance phenomenon.

REFERENCES

1. Reutter V, Kottgen E, Bauer C, Geroke W: Biological significance of sialic acids, in Schauer R (ed): Sialic Acids: Chemistry, Metabolism and Function. New York, Springer-Verlag, 1982, p 263

2. Baker MA, Taub RN, Whelton CH, Hindenburg A: Aberrant sialylation of granulocyte membranes in chronic myelogenous leukemia. Blood 63:1199, 1984

3. Van Beek WP, Smets LA, Emmelot P: Increased sialic acid density in surface glycoprotein of transformed and malignant cells—A general phenomenon? Cancer Res 33:2913, 1973

4. Lotan R, Lotan D, Meromsky L: Correlation of retinoic acid–enhanced sialytransferase activity and glycosylation of specific cell surface sialoproteins with growth inhibition in a murine melanoma cell system. Cancer Res 44:5805, 1984

5. Brown AE, Schwartz EL, Breyer RN, Sartorelli AC: Synthesis of sialoglycoconjugates during dimethylsulfoxide-induced erythrodifferentiation of Friend leukemia cells. Biochem Biophys Acta 714:217, 1982

6. Durham JP, Ruppert M, Fontana JA: Glycosyltransferase activities and the differentiation of human promyelocytic (HL-60) cells by retinoic acid and phorbol ester. Biochem Biophys Res Commun 110:348, 1983

7. Liv C-K, Schmied R, Schreiber C, Rosen A, Qian G-X, Waxman S: Glycosyltransferase alterations are cell type related when human promyelocytic leukemia (HL-60) cells are treated with various inducers of differentiation. Exp Hematol 11:738, 1983

8. Nojiri H, Takaken F, Tetsuka T, Saito M: Stimulation of sialidase activity during cell differentiation of human promyelocytic leukemia cell line HL-60. Biochem Biophys Res Commun 104:1239, 1982

9. Bosmann HB: Mechanism of cellular drug resistance. Nature 23:556, 1971

10. Hindenburg A, Bhalla K, Taub RN, Grant S, Baker MA: Decreased membrane sialylation of Adriamycin-resistant HL-60 leukemia cells. Blood 62:160a, 1983 (suppl 1) (abstract).

11. Gallagher RE, Ferrari AC, Zulich AW, Yen R-WC, Testa JR: Cytotoxic and cytodifferentiative components of 6-thioguanine resistance in HL-60 cells containing acquired double minute chromosomes. Cancer Res 44:2642, 1984

12. Gallagher RE, Bilello PA, Ferrari AC, Chang C-S, Yen R-WC, Nickols WA, Muly EC III: Characterization of differentiation-inducer–resistant HL-60 cells. Leuk Res 9:967, 1985

13. Felsted RL, Gupta SK, Glover CJ, Gallagher RE: Surface membrane glycoproteins of wild-type and differentiation-inducer–resistant HL-60 cells. Blood 66:606, 1985

14. Spiro RG: Glycoproteins. Annu Rev Biochem 39:599, 1970

15. Warren L: Tiobarbituric acid assay of sialic acids, in Colowick SF, Kaplan NO (eds): Methods in Enzymology (vol 6). Orlando, FL, Academic, 1963, p 463

16. Roboz J, Suttajit M, Bekesi JG: Elimination of 2-deoxyribose interference in the biobarbituric acid determination of *N*-acetylneuraminic acid in tumor cells by pH-dependent extraction with cyclohexanone. Anal Biochem 10:380, 1981

17. Bradford M: A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976

18. Felsted RL, Gupta SK, Glover CJ, Fischkoff SA, Gallagher RE: Cell surface membrane protein changes during the differentiation of cultured human promyelocytic leukemia cells. Cancer Res 43:2754, 1983

19. Jourdian GW, Dean L, Roseman S: The sialic acids XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. J Biol Chem 246:430, 1971

20. Baumann H, Doyle D: Localization of membrane glycoproteins by *in situ* neuraminidase treatment of rat hepatoma tissue culture and two-dimensional gel electrophoretic analysis of the modified proteins. J Biol Chem 254:2542, 1979

21. Hindenburg AA, Taub RN, Grant S, Chang G, Baker MA: Effects of pyrimidine antagonists on sialic acid regeneration in HL-60 cells. Cancer Res 45:3048, 1985

22. Schneider C, Owens MJ, Banville D, Williams JG: Primary structure of human transferrin receptor deduced from the mRNA sequence. Nature 311:675, 1984

23. Gahmberg CB, Andersson LC: Role of sialic acid in the mobility of membrane proteins containing O-linked oligosaccharides on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Eur J Biochem 122:581, 1982

24. Kartner N, Shales M, Riordan JR, Ling V: Daunorubicin-resistant Chinese hamster ovary cells expressing multidrug resistance and a cell surface P-glycoprotein. Cancer Res 43:4413, 1983

25. Peterson RHF, Myers MB, Spengler BA, Biedler JL: Alteration of plasma membrane glycopeptides and gangliosides of Chinese hamster cells accompanying development of resistance of daunorubicin and vincristine. Cancer Res 43:222, 1983

CONCISE REPORT

In Vitro Tumor Cell Cytolysis Mediated by Peptide Defensins of Human and Rabbit Granulocytes

By Alan Lichtenstein, Tomas Ganz, Michael E. Selsted, and Robert I. Lehrer

We examined the activity of defensins, cysteine-rich cationic peptides that are abundant in the cytoplasmic granules of human and rabbit granulocytes, against various tumor targets. The three human defensins, HNP-1, HNP-2, and HNP-3, lysed human and murine targets in chromium release and dye exclusion assays. Defensin-mediated tumor cell lysis was concentration-dependent, inhibited by serum, and dependent on temperature-sensitive events. Lysis was first detected by three hours of incubation and it reached a plateau between eight and 14 hours. In vitro exposure of murine teratocarcinoma cells to HNP 1-3 abrogated their oncogenicity in vivo. Nonmalignant target cells were also susceptible to defensin-mediated lysis. Four rabbit granulocyte defensins exerted marked (NP-1, NP-2) or moderate (NP-3a, NP-3b) cytotoxic activity, whereas defensin NP-5 was not cytotoxic. When tumor cells were incubated with human defensins in combination with hydrogen peroxide, synergistic cytotoxicity was detected. As defensins are released from granulocytes by various stimuli, their release could contribute to extracellular cytotoxicity which is independent of reactive oxygen intermediates.

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TWO DISTINCT TYPES of neutrophil-mediated tumor lysis have been described. One of these is linked to the generation of reactive oxygen intermediates (ROIs)[1-3] and is analogous to the O_2-dependent microbicidal mechanisms of the polymorphonuclear cell (PMN). However, PMNs from subjects with chronic granulomatous disease, despite their inability to generate ROIs, show substantially intact lytic activity against various targets[4-6] under some conditions. The mechanisms of ROI-independent cytotoxicity are less well understood, but at least one component of the PMN's azurophil granule, a chymotrypsinlike cationic protein,[7] has been shown to exert cytotoxicity against a murine lymphoma cell target.

Human and rabbit PMNs contain substantial amounts of defensins, small (molecular weight ~3,500) cysteine- and arginine-rich cationic peptides[8,9] which exert potent antimicrobial[10,11] and antiviral[11,12] activity. The human defensins are released from PMNs exposed to secretagogues such as phorbol myristate acetate and opsonized zymosan.[13] Two rabbit defensins, NP-1 and NP-2, initially isolated from lung macrophages, were recently shown to exert selective cytotoxicity against murine lymphoma cells.[14] We now report on cytotoxicity of eight defensins, five obtained from rabbit PMNs and three from human cells.

MATERIALS AND METHODS

Purification of defensins. Rabbit defensins NP-1, NP-2, NP-3a, NP-3b and NP-5, were purified by a modification of our previously described protocol.[10] Briefly, 1 to 2 × 10^{10} peritoneal PMNs were disrupted by homogenization, extracted five times with 10% acetic acid at 8 °C and centrifuged for 20 minutes at 27,000 × g. Supernatants were pooled, lyophilized, resuspended in 30 mL of 10% acetic acid and subjected to gel filtration on a 4 × 150 cm Bio-Gel P-10 column equilibrated in 5% acetic acid. Defensin-containing fractions were pooled, lyophilized, and then separated by cation-exchange high-performance liquid chromatography (HPLC) on a 21.5-x-150-mm Bio-Sil TSK-CM-3-SW column. The sample was dissolved and applied to the column, in buffer A (0.05 mol/L sodium phosphate, pH 6.7, 10% acetonitrile) and eluted by application of a linear gradient of buffer B (0.05 mol/L sodium phosphate, pH 6.7, 3.5 mol/L sodium chloride, 10% acetonitrile). A 0% to 50% gradient was developed over 80 minutes at a flow rate of 6 mL/min. Near-baseline separation of the individual peptides was obtained in this step. Final purification was achieved by reverse-phase HPLC as described previously.[10] The human defensins HNP-1, HNP-2, and HNP-3 were obtained from normal PMNs and purified to homogeneity as previously described,[11] by applying sequential ion-exchange and reverse-phase HPLC followed by gel exclusion chromatography on a long Bio-Gel P-10 column. Lyophilized defensins were dissolved in 0.01% acetic acid at 1 mg/mL and stored at −20 °C until used.

Tumor lines. Suspension cultures of human (Raji, MOLT-4, K562, IM-9, U-937, and WIL-2) and murine (P815, YAC-1) lines were maintained in RPMI medium with 10% fetal calf serum (FCS, Reheis, Phoenix, AZ). The MOT teratocarcinoma was maintained in ascites form in C3H mice. The YAC-R[15], U9TR, and U9NR[16] lines were gifts from Dr S. Wright. YAC-R and U9NR, cloned from parental YAC-1 and U937 lines, had been rendered specifically resistant to lysis by natural killer cytolytic factor (NKCF). U9TR had been rendered specifically resistant to lysis by tumor necrosis factor (TNF).

Chromium release assay (CRA). Targets (10^7) were incubated with 50 μCi ^{51}Cr for one hour at 37 °C in RPMI-10% FCS. Labeled targets (10^4 in 0.1 mL of RPMI-1640) and the specified defensins (in 0.1 mL of RPMI) were incubated in microtitre plates at 37 °C in 5% CO_2. Subsequently, the plates were centrifuged (1,200 rpm × 10 min) and 0.1 mL of supernatant was counted in a gamma counter.

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Percent lysis was calculated as: $(\text{cpm}_{\text{exp}} - \text{cpm}_{\text{control}})/(\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{control}}) \times 100$. Control release was determined from targets incubated in media alone (<35% incorporated counts), and maximal release was achieved by adding 1 mol/L HCl (>80% incorporated counts). Release from targets incubated in RPMI containing dilute acetic acid (the vehicle used in defensin stock solutions) did not differ from controls incubated in RPMI alone. The standard deviation (SD) of triplicate samples was always <5% of the mean.

Dye exclusion assay. Targets (10^4) in 0.1 mL RPMI were incubated with 0.1 mL of defensins or in medium alone (control wells). Samples were run in quadruplicate in microtitre plates. The plates were incubated in 5% CO_2 at 37 °C and surviving targets were counted by trypan blue exclusion. Percent cytotoxicity was calculated as: (mean no. of viable targets in control wells) − (mean no. of viable targets in test wells)/(mean no. targets in control wells) × 100.

Oncogenicity assay. MOT cells (2×10^6) were incubated in 5 mL of RPMI with 0.01% acetic acid (control) or in RPMI with 100 μg/mL of HNPs 1-3. After eight hours in 5% CO_2 at 37 °C, MOT cells were washed, resuspended, and aliquots were injected IP into C3H mice (female, 8 wks old, eight mice/group). Each mouse received 0.5 mL of 10^3 or 10^4 cells on day 0.

Use of nonmalignant cells as targets. Murine thymocytes and spleen cells were obtained from adult C3H mice as previously described.[17] Peripheral blood PMNs and lymphocytes were harvested from gradients as previously described.[18] Viability of targets was >90%.

RESULTS

Tumor lines incubated with a mixture of the three human defensins (HNP 1, 2, and 3 in a ratio of 2:2:1 by weight) for six hours at 37 °C demonstrated significant isotope release (Table 1). Tumor lysis was concentration-dependent and optimal lysis was achieved with 25 to 100 μg/mL. Table 1 also demonstrates the lytic potency of HNPs in dye-exclusion assays, wherein cytotoxicity (in parentheses) was also concentration-dependent for WIL-2 and K562 targets. The CRAs in Table 1 were performed in the absence of serum. The presence of 1% FCS decreased HNP-mediated lysis by 30% and the presence of 5% FCS decreased it by 75%. Lysis was also strictly dependent upon temperature-sensitive events (optimal lysis at 37 °C; no lysis at 25 °C).

Although their lysis was always significant, murine lines were consistently less sensitive than human lines as targets (Table 1). The order of sensitivity of human lines shown in Table 1 (WIL-2 > IM-9 > RAJI >K562 >MOLT-4) was consistently present in each of five experiments. Variant tumor lines, specifically resistant to NKCF (YAC-R, U9NR) or TNF (U9TR) were equally as sensitive as their respective parent lines to HNP-mediated lysis. Exposure of MOT cells to HNPs also destroyed their in vivo oncogenicity. Mice inoculated with tumor cells (10^3 or 10^4/group; eight mice/group) incubated in control media all died within 35 days after a period of progressive ascites. In contrast, MOT cells exposed to 100 μg/mL of HNPs 1-3 caused tumor outgrowth in three of eight mice injected with 10^4 MOT cells, and in none of eight injected with 10^3 cells.

Significant chromium release was not detected until three hours of incubation and rose to very high levels by eight hours (Fig 1). Whereas a plateau was reached in eight hours for some targets, lysis of others continued to increase linearly until 14 hours of incubation. Spontaneous release exceeding 35% precluded assaying groups past 14 hours of incubation.

Both dye exclusion and CRA confirmed HNP-mediated lysis of nonmalignant targets. Murine thymocytes and spleen cells as well as human lymphocytes and PMNs were sensitive to lysis and this was comparable to corresponding tumor cells (data not shown).

To examine the correlation between lysis and net charge of the defensins, each of the human peptides were tested separately and compared to similarly purified rabbit PMN peptides (Table 2). Within each species, the degree of lysis roughly correlated with the overall antimicrobial effects of the individual peptides,[10,11] but there was little correlation with cationic charge. Note that although rabbit NP-5 was more cationic than any of the human defensins, it was

Table 1. Lysis of Various Tumor Lines by Defensins*

		Concentration of Defensins (mcg/mL)				
	Target	100	25	10	1	0.1
Human						
	Wil-2	94 ± 4 (98 ± 4)	91 ± 6 (93 ± 3)	43 ± 4 (72 ± 5)	25 ± 2 (45 ± 4)	3.5 ± 0.2
	IM-9	88 ± 4.1	—	—	—	—
	Raji	80 ± 4	71 ± 4.2	29 ± 2.7	15 ± 2.1	0.7 ± 0.1
	K562	54 ± 3 (91 ± 5)	48 ± 2 (74 ± 6)	15 ± 1.4 (49 ± 4)	8.7 ± 0.6 (35 ± 3)	0.4 ± 0.2
	MOLT-4	48 ± 2.4	—	—	—	—
	U937	46 ± 2.7	39 ± 3.2	28 ± 3.1	14 ± 1.5	—
	U9NR†	44 ± 3.1	35 ± 2.7	25 ± 2.2	13 ± 1.2	—
	U9TR‡	49 ± 4	37 ± 3	29 ± 3	15 ± 1.6	—
Mouse						
	YAC-1	35 ± 4.2	27 ± 4	15 ± 1.5	6 ± 1	—
	YAC-R†	38 ± 2.5	29 ± 2.1	15 ± 2.1	5 ± 0.8	—
	MOT	30 ± 2.6	—	—	—	—
	P815	27 ± 1.7	—	—	—	—

*Tumor cells incubated with varying concentrations for six hours; specific lysis determined by CRA; figures in parentheses denote cytotoxicity, determined in dye exclusion assay, for Wil-2 and K562; mean ± SD of five separate experiments.

†Tumor line resistant to NKCF.

‡Tumor line resistant to TNF.

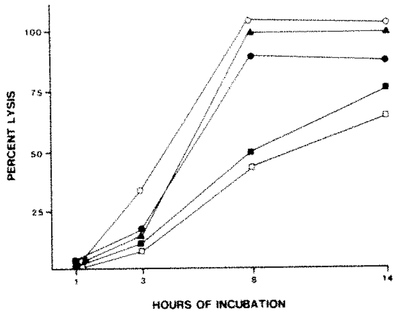

Fig 1. Kinetics of lysis by defensins. Tumor lines incubated with defensins for one, three, eight, or 14 hours and chromium-release–assayed. O—O, WIL-2; ▲—▲, IM-9; ●—●, RAJI; ■—■, K562; □—□ MOLT-4.

ineffective as a lytic agent. Also, NP-2 is more lytic than NP-3a and NP-3b and carries the same charge.

Although a cationic charge is not sufficient to induce lysis, CRAs performed in the presence of the polyanion substance heparin suggest that net charge plays a role in the detected cytolysis. Defensin-mediated lysis decreased by 63% and 92% in the presence of 1 U/mL and 10 U/mL heparin, respectively.

Since defensins might be secreted by PMNs in tandem with H_2O_2, we next examined possible interactions between these two potential cytolysins. When K562 targets are incubated with reagent grade H_2O_2 in combination with 20 or 40 µg/mL of HNPs 1-3, tumor lysis is significantly greater than the sum of that achieved by either agent alone (Fig 2). This was most evident between H_2O_2 concentrations of 10^{-5} and 10^{-3} mol/L. Sodium azide (1 mmol/L) did not prevent synergistic killing, ruling out the possibility that the HNP preparation was contaminated with trace amounts of myeloperoxidase that could have reacted with H_2O_2 and augmented cytolysis.

DISCUSSION

Several studies have demonstrated that PMNs can lyse target cells by mechanisms not involving ROI production.

Table 2. Comparison of Lysis by Individual Rabbit and Human Defensins

			% Specific lysis*	
	Peptide	Net Charge	100 mcg/mL	25 mcg/mL
Human	HNP-1	+3	55.0 ± 2.3	27.0 ± 2.8
	HNP-2	+3	43.5 ± 2.3	26.2 ± 2.4
	HNP-3	+2	22.5 ± 2.7	22.3 ± 2.6
Rabbit	NP-1	+9	84.3 ± 3.2	70.1 ± 2.6
	NP-2	+8	82.7 ± 2.5	68.3 ± 3.9
	NP-3a	+8	26.7 ± 0.7	9.5 ± 1.0
	NP-3b	+8	36.5 ± 3.5	16.1 ± 1.3
	NP-5	+4	5.1 ± 1.1	0.9 ± 1.1

Individually purified human or rabbit defensins, 25 or 100 mcg/mL, were incubated with ^{51}Cr-labelled K562 targets for eight hours.

*Specific lysis is shown as the mean ± S.D. of three separate experiments. Net cationic charge at pH 7.4 was calculated from the published amino acid sequences of the defensins (8, 9), as follows: Net charge = [(arginine + lysine residues) − (glutamic acid + aspartic acid residues)].

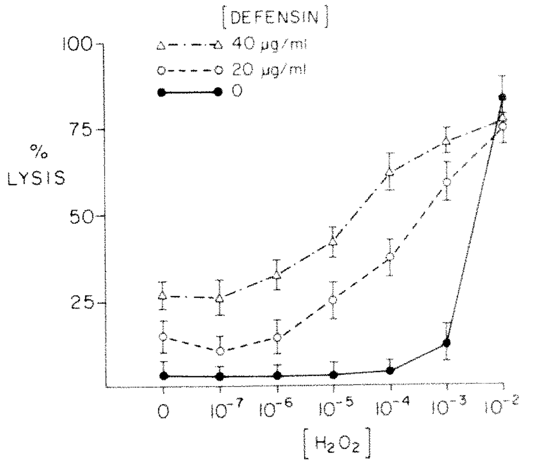

Fig 2. Synergistic lysis of tumor cells. Chromium-labeled K562 targets incubated for four hours with H_2O_2 (10^{-7} to 10^{-2} mol/L), defensins (20 or 40 µg/mL) or both agents. Percent lysis determined after incubation and presented as mean ± SD of quadruplicate samples.

The present study demonstrates that defensins, recently characterized antimicrobial peptides of human and rabbit PMNs, can damage various normal and tumor cell targets in vitro. Defensin-mediated lysis was confirmed in three independent assays: isotope release, dye exclusion and in vivo oncogenicity. The presence of cytotoxic components such as defensins in the cytoplasmic granules of the PMN echoes the presence of other cytotoxic constituents in natural killer (NK) lymphocytes[19] and certain macrophages.[20] It is possible, but yet unproven, that secretion of cytotoxic principles such as HNPs from PMNs underlies their ability to lyse adjacent targets. In addition, the data of Fig 2 suggest that small amounts of defensins may act synergistically with H_2O_2 to achieve heightened tumor lysis.

The mechanism of lysis is not yet known. The temporal delay in its onset (Fig 1) and its prominent temperature dependence suggest that more than a simple detergent effect is operative. The small size of the defensins excludes their functioning as enzymes such as the cytolytic serine protease reported from cytotoxic macrophages.[21] The undiminished sensitivity of tumor cells resistant to NKCF or TNF suggests that the mechanism differs qualitatively from that induced by those two cytotoxins.

Other cationic proteins are often cytotoxic.[22] In addition, the presence of the polyanion heparin prevented HNP-mediated lysis, suggesting that charge of these molecules plays a role in target cytotoxicity. However, Table 2 indicates that the lytic effect of HNPs is not solely due to their cationicity as there was a poor correlation between charge and cytotoxicity of the individual defensins.

In contrast to the effects of rabbit macrophage defensins,[14] the human defensins did not show selective cytotoxicity for malignant targets. The use of less potent cytotoxic material in a longer assay[14] may have allowed the detection of selective cytotoxicity. In addition, the prerequisite for antibody-directed effector: target binding in PMN-mediated antibody-dependent cellular cytotoxicity (ADCC), as well as the tendency of inflammatory PMNs to bind to neoplastic targets more effectively than to nonmalignant targets, even in the absence of antibody,[23] could impart target specificity to an otherwise nonspecific molecular effector.

Serum markedly inhibited the ability of HNPs to effect cytotoxicity. We attribute this to avid binding of HNPs by albumin and other macromolecular serum components (M. Selsted, unpublished data, April 1986). This suggests that HNP-mediated lysis might only occur if tight binding of targets to PMNs creates a contact zone that excludes macromolecular serum components. This possibility is currently under investigation.

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REFERENCES

1. Nathan CF, Brukner L, Silverstein S, Cohn ZA: Lysis of tumor cells by activated macrophages and granulocytes. J Exp Med 149:84, 1979
2. Nathan CF, Silverstein S, Brukner L, Cohn ZA: Extracellular cytolysis by activated macrophages and granulocytes. J Exp Med 149:100, 1979
3. English D, Lukens J: Regulation of neutrophil mediator release: Chemotactic peptide activation of stimulus-dependent cytotoxicity. J Immunol 130:850, 1983
4. Dallegri F, Patrone F, Frumento G, Sacchetti C: Antibody-dependent killing of tumor cells by polymorphonuclear leukocytes. Involvement of oxidative and non-oxidative mechanisms. JNCI 73:331, 1984
5. Katz P, Simone C, Henkart P, Fauci AS: Mechanisms of antibody-dependent cellular cytotoxicity. The use of effectors from chronic granulomatous disease patients as investigative probes. J Clin Invest 65:55, 1980
6. Siebens H, Tevethia SS, Babior BM: Neutrophil-mediated antibody-dependent killing of herpes-simplex-infected cells. Blood 54:88, 1979
7. Clark RA, Olsson I, Klebanoff SJ: Cytotoxicity for tumor cells of cationic proteins from human neutrophil granules. J Cell Biol 70:719, 1976
8. Selsted ME, Brown DM, LeLange RJ, Harwig SL, Lehrer RI: Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. J Biol Chem 260:4579, 1985
9. Selsted ME, Harwig SL, Ganz T, Schilling JW, Lehrer RI: Primary structures of three human neutrophil defensins. J Clin Invest 76:1436, 1985
10. Selsted ME, Szklarek D, Lehrer RI: Antimicrobial properties of rabbit granulocytes: Purification and antimicrobial activities. Infect Immun 45:150, 1984
11. Ganz T, Selsted ME, Szklarek D, Harwig SL, Daher K, Bainton DF, Lehrer RI: Defensins: Natural peptide antibiotics of human neutrophils. J Clin Invest 76:1427, 1985
12. Lehrer RI, Daher K, Ganz T, Selsted ME: Direct inactivation of viruses by MCP-1 and MCP-2, natural peptides from rabbit leukocytes. J Virol 54:546, 1985
13. Ganz T, Cardaci C, Selsted ME, Lehrer RI: Secretion of antimicrobial defensins by human neutrophils. Clin Res 34:458A, 1986 (abstr)
14. Sheu MJ, Baldwin WW, Brunson KW: Cytotoxicity of rabbit macrophage peptides MCP-1 and MCP-2 for mouse tumor cells. Antimicrob Agents Chemother 28:626, 1985
15. Wright S, Bonavida B: YAC-1 clones selected for resistance to natural killer cytotoxic factors are resistant to natural killer cell-mediated cytotoxicity. Proc Natl Acad Sci USA 80:1688, 1983
16. Wright S, Bonavida B: Evidence for the involvement of human natural killer cytotoxic factors but not tumor necrosis factor in the NK lytic mechanisms. Nat Immun Cell Growth Regul 4:287, 1985
17. Lichtenstein A, Mickel R, Zighelboim J: Inhibition of natural killer cell lysis by natural killer-inhibitory substance. Cell Immunol 80:66, 1983
18. Lichtenstein A, Naiem F, Zighelboim J: Peripheral blood lymphocytes with receptors for a determinant common to B-lymphoblastoid cell lines and AML blasts. Am J Hematol 16:383, 1984
19. Millard PJ, Henkart M, Reynolds CW, Henkart P: Purification and properties of cytoplasmic granules from cytotoxic rat LGL tumors. J Immunol 132:3197, 1984
20. Hibbs JB: Heterocytolysis by macrophages activated by BCG: Lysosomal exocytosis into tumor cells. Science 184:468, 1974
21. Adams DO, Kao K-J, Farb R, Pizzo SV: Effector mechanisms of cytolytically activated macrophages. J Immunol 124:293, 1980
22. Antohi S, Brumfeld V: Polycation-cell surface interactions and plasma membrane compartments in mammals. Interference of oligocation with polycationic condensation. Z Naturforsch 39C:767, 1984
23. Lichtenstein A, Kahle J, Bonavida B: Use of a self-generating percoll gradient and cytotoxicity assay to identify tumor-lytic properties of inflammatory neutrophils. J Immunol Methods 81:95, 1985

CONCISE REPORT

Human Myeloperoxidase Gene: Molecular Cloning and Expression in Leukemic Cells

By Kun Sang Chang, Jose M. Trujillo, Richard G. Cook, and Sanford A. Stass

We have molecularly cloned the human myeloperoxidase (MPO) gene from the λgt11 expression library by screening with an affinity-purified MPO antibody. The cDNA clone of the MPO gene was used to study MPO gene expression in leukemic cells. The amino acid sequence predicted from the nucleotide sequence of the cDNA clone pMP401 matched exactly the 23 amino acid sequence of the NH_2-terminal of the 60,000 MPO subunit. We found that MPO cDNA hybridized to a single *Eco*RI genomic band of 19 kb, indicating that the MPO gene represents a single gene in the human genome. Northern blot analysis of RNA isolated from leukemic cell lines and acute myelogenous leukemia (AML) patients' samples shows that MPO gene expression correlated with myeloid lineage. The intensity of MPO mRNA expression on Northern blot correlated with the level of MPO expression by cytochemical staining. Multiple species of MPO mRNA were found. This indicates that a single MPO gene may encode different RNA species through a mechanism of posttranscriptional processing or that multiple transcriptional start/termination sites exist in the MPO gene.

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HUMAN MYELOPEROXIDASE (MPO) is a hemoprotein and glycoprotein found in primary granules of the myeloid series and is considered to be the definitive marker of myeloid lineage. Acute leukemias that express greater than 3% MPO in the blasts are usually classified as acute myelogenous leukemia (AML) regardless of other phenotypic markers. The MPO enzyme has potent bacteriocidal activity in the presence of H_2O_2 and a halide ion. This enzyme has been purified and characterized in several different laboratories,[1-3] and appears to have a molecular weight in the range of 150,000. It is generally accepted that MPO consists of two large subunits with a molecular weight of 60 kd and two smaller molecular weight subunits of 15 kd. Pulse label experiments indicated that both large and small subunits of the MPO protein are derived from the same precursor polypeptides of 81 kd and 89 kd through a mechanism of posttranslational modification.[4] These observations indicate that both subunits are encoded by the same MPO gene. Posttranslational modifications may also account for the finding that three different but very similar MPO protein forms can be isolated and purified to homogeneity.[2,3] The MPO size heterogeneity could be due to the slightly different amino acid compositions of these three proteins or a difference in sugar moieties.

We have molecularly cloned the human MPO cDNA gene. Our studies provide new evidence that a single MPO gene may be responsible for the transcription of multiple forms of MPO messenger RNA, indicating that posttranscriptional modification may be involved in the processing of the MPO mRNA or that multiple transcriptional start or termination sites exist in the MPO gene.

MATERIALS AND METHODS

Total RNA was isolated from myeloperoxidase-positive leukemic blasts and promyelocytes obtained from a patient with AML using guanidine isothiocyanate.[5] Poly A+ RNA was selected from total RNA[6] and double-stranded cDNA was synthesized as described.[7] A cDNA expression library was constructed in a λgt11 vector (Vector Cloning System, San Diego, CA).[8]

MPO antiserum was raised in a rabbit using purified human leukocyte MPO (RZ value not less than 0.84) (Green Cross, Osaka, Japan). The IgG fraction of the crude MPO antiserum was selected by passing through a DEAE-Affi-Gel Blue column (Bio-Rad, Richmond, CA). MPO-specific antibody was affinity-purified by incubating the IgG fraction with MPO protein–bound nitrocellulose strips (10 μg MPO/cm²). The filter strips were thoroughly washed and MPO-specific antibody was eluted with low pH buffer and neutralized as described.[9] This affinity-purified MPO antibody was further treated with *E coli* lysate (Bio-Rad) to eliminate any background signal during screening. The resulting MPO antibody was shown to immunoprecipitate MPO and was used to screen the λgt11 library. The library was screened as described by the manufacturer using the Express Blot Kit (Bio-Rad).

Positive clones were purified and the phage DNA was isolated. The cDNA clones were excised by *Eco*RI digestion and subcloned into either pUC18 or pBR322 plasmid on the *Eco*RI site.[10]

DNA sequences from each end of the cDNA inserts were determined by the Maxam and Gilbert method.[11] The 60-kd, 39-kd, and 15-kd MPO protein subunits were isolated[12] and the amino acid sequences were determined by an Applied Biosystems 470A gas phase protein sequencer. Phenylthiohydantoin (PTH) amino acids were identified by high-performance liquid chromatography using the applied biosystem 120A PTH analyzer. Approximately 200 to 250 pmol of protein were sequenced with a repetitive yield of 92%. DNA or amino acid sequence homology was compared with the aid of an Apple IIc computer and the software of R. Larson and J. Messing (University of Minnesota, Minneapolis, Minn).

Genomic DNA and RNA were extracted from blood samples and cultured cell by the guanidinium isothiocyanate and cesium chloride (CsCl) centrifugation procedure.[5] RNA was pelleted at the bottom of the centrifuge tube, and genomic DNA was collected at the upper part of the CsCl and dialyzed extensively before restriction endonu-

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clease digestion. Southern and Northern blot analysis was performed as previously described.[10]

RESULTS

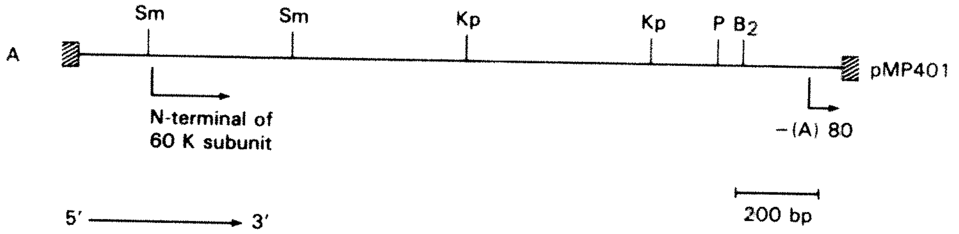
The λgt11 cDNA library was screened with the affinity-purified MPO antibody. cDNA clones producing fusion proteins were identified by their strong reaction with MPO-specific antibody. These λ phage cDNA clones were plaque-purified to 100% positivity and the recombinant phage DNAs were prepared by a minilysate procedure.[10] From 0.8 mL of phage lysate we routinely are able to obtain enough recombinant DNA for mini-gel analysis after *Eco*RI digestion and subcloning of the digested cDNA insert into a plasmid vector. All clones were found to carry a cDNA insert which varied from 1.8 kb to 3.2 kb (data not shown). No internal *Eco*RI sites were found in any clone. These cDNA subclones were grown to obtain large quantities of plasmid DNA. cDNA inserts were excised and purified for DNA sequence analysis. We found that the DNA sequence of clone pMP401 as shown in Fig 1A could be translated into an amino acid sequence obtained from the NH_2-terminal of 60-kd–molecular weight subunit of the MPO protein (Fig 1B, 1C, and 1D). This result proves unambiguously that pMP401 is the cDNA clone of the human myeloperoxidase gene. The 3′ end of the gene was identified by a stretch of a 80-bp poly A tail. pMP401 carries a cDNA insert of 1.85 kb (Fig 1A). It has enough gene sequence capacity to encode for the entire 60-kd protein subunit, but not both the 60-kd and 15-kd subunits. In addition, the 180 bp of DNA sequence beyond the NH_2-terminal of the 60-kd subunit did not show any ATG or translational start codon. These observations lead us to conclude that the 15-kd MPO protein subunit was encoded on the 5′ region of the MPO mRNA. An alternative possibility is that part of the MPO gene may have been deleted during the cloning procedure.

Southern blot analysis of the genomic DNA shown in Fig 2 indicates that the MPO gene represents a single gene in the human genome, since only a single band of 19 kb was observed when the genomic DNA was digested with *Eco*RI. No rearrangement of the MPO gene was seen using three different restriction digests (*Hind*III, *Eco*RI, and *Bam*HI) of DNA from leukemic blasts of patient 1 with AML, patient 3 with chronic myelogenous leukemia in lymphoblastic crisis, and cell lines 8402 and HL-60. The difference in *Bam*HI restriction fragment size in 8402 DNA is due to the result of partial digest.

Northern blot analysis of total RNA isolated from leukemic blasts of patient 1 with AML revealed multiple MPO mRNA bands (Fig 3). At least two equally strong signals were observed between a 3.1- to 3.3-kb region. Two additional weaker bands were also seen at the 2.5- and 2.7-kb region. The signal for patient 2 and HL-60 was not sufficiently intense to determine if multiple mRNA species exist. Since MPO represents a single gene in the human genome, the finding of multiple bands in patient 1 indicates that there may be posttranscriptional modification of the MPO mRNA, resulting in the generation of different mRNA sizes, or there may be multiple transcriptional starts or termination sites.

Figure 3 also shows that the MPO gene was not expressed in MPO-negative lymphoblastic cell line 8402. Patient 1 showed high levels of MPO gene activity, HL-60 expressed

A



B

```
GLY VAL ASN CYS GLU THR SER CYS VAL GLN GLN PRO PRO CYS PHE PRO LEU LYS ILE PRO
GGC GTC AAC TGC GAG ACC AGC TGC GTT CAG CAG CCG CCC TGC TTC CCG CTC AAG ATC CCG

PRO ASN ASP PRO
CCC AAT GAC CCC
```

C

```
                                   10                                          20
VAL ASN CYS GLU THR SER CYS VAL GLN GLN PRO PRO CYS PHE PRO LEU LYS ILE PRO PRO

                                   30                                          40
ASN ASP PRO ARG ILE LYS ASN GLN ALA ASP
```

D

```
         71                   81                   91
VNCETSCVQQPPCFPLKIPPNDP
***********************
VNCETSCVQQPPCFPLKIPPNDPRIKNQAD
         10                   20                   30
```

Fig 1. (A) Restriction map of the MPO cDNA clone pMP401. Location of the NH_2-terminal coding region of the 60-kd subunit and a stretch of 80-bp poly A tail are shown. Restriction endonucleases sites were as follows: Sm, *Sma*I; Kp, *Kpn*I; P, *Pst*I; B_2, *Bgl*II. (B) Nucleotide sequence and the predicted amino acid sequence at the 5′ end of pMP401. (C) The amino acid sequence of the NH_2-terminal of the MPO 60-kd subunit. (D) Comparison of the amino acid sequences as shown in (B) and (C). Asterisks indicate identical amino acids.

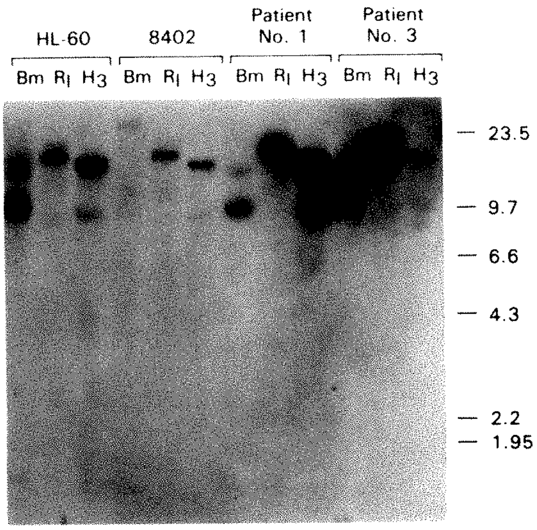


Fig 2. Southern blot analysis of genomic DNAs—patient 1, AML; patient 3, CML, lymphoid blast crisis; lymphoblastic cell line 8402; and HL-60. Restriction endonuclease digests were as follows: Bm, *Bam*HI; R_I, *Eco*RI; H_3, *Hind*III. DNA size markers indicated on the right are λ DNA *Hind*III-digested fragments. The filter was hybridized to ^{32}P-labeled probe of pMP401. The pMP401 plasmid was oligolabeled to 10^9 cpm/μg DNA as described by Feinberg and Fogelstein.[13]

the MPO gene at a much lower level, and patient #2 showed very low levels of MPO gene expression. This data correlated with the MPO expression as determined by light microscopic cytochemical staining (pt 1, >90%; pt 2, 9%).

DISCUSSION

The MPO protein has been extensively studied. However, due to the complexity of the protein subunits, many questions concerning the structure and mechanism of enzyme action are unanswered. The cDNA clone for MPO provides a powerful tool to investigate MPO structure and function. Using our cloned human leukocyte MPO cDNA we have found that it represents a single gene in the human genome (Fig 2). This contradicts the finding at the protein level which showed multiple purified forms of MPO and slightly different molecular weights for the 60-kd subunit.[2,3] Our Northern blot analysis (Fig 3) indicates that there are multiple species of MPO mRNA present in AML and HL-60. It has been shown that posttranslational processing is involved in the generation of the large and small subunit of the MPO protein.[4] Our findings suggest that posttranscriptional processing of the MPO mRNA or multiple transcriptional start/termination sites on the mRNA result in different mRNA species which may play an important role in the assembling of the MPO protein into different forms. Such mechanisms for gene regulation have been previously described.[14,15] The NH_2-terminal amino acid sequences of the 60-kd MPO subunit (Fig 1) match exactly the 23 amino acids predicted from the MPO gene nucleotide sequence. We have found that the 39-kd subunit isolated under nonreducing conditions as described by Nauseef and Malech[12] have completely different NH_2-terminal amino acid sequences (data not shown). According to the peptide map of the 60-kd and 39-kd subunit presented by the authors, most fragments between the two subunits are identical except for two fragments that were missing in the 39-kd subunit. This observation together with our sequencing data seems to suggest that the 39-kd subunit is part of the 60-kd subunit, and that a 20-kd polypeptide was lost under nonreducing conditions. Final proof of these observations will depend on the complete sequencing of the full-length MPO cDNA.

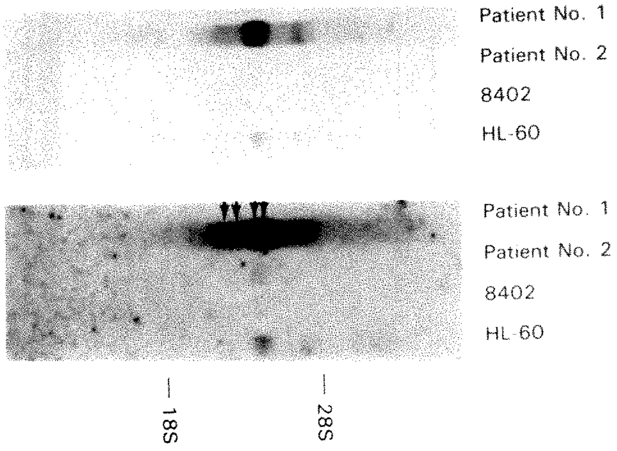


Fig 3. Expression of myeloperoxidase gene in human leukemic cells. Total RNAs (15 μg) were loaded on each lane and separated electrophoretically in 1% agarose. Patient #1, AML; patient 2, AML; 8402; and HL-60. Locations of 18S and 28S RNA are indicated on the right. Right panel represents a longer exposure and shows four mRNA bands (indicated by arrows) in patient 1. Hybridization probe was prepared as described in Fig 2.

As previously reported, three different forms of MPO exhibited slightly different properties.[2] Form I had a different substrate specificity compared to Forms II and III and they are likely to be compartmentalized. Thus, each of these forms may have a different cellular function and may be expressed in a specific type of myeloid cell. To investigate this possibility, cDNA clones that represent different mRNA species may be used to identify specific regions in the gene that do not hybridize to other MPO mRNA species. These specific regions can then be used to perform in situ hybridization to localize each mRNA species in specific myeloid cells. It will also be necessary to study the natural (genomic) gene of MPO in order to understand the mechanism that controls the expression of the different mRNA species. It will be important to determine whether blasts in leukemic patients that are negative for MPO by light and ultrastructure show evidence of MPO mRNA. This may further refine our ability to detect the earliest stage of myeloid lineage commitment.

ACKNOWLEDGMENT

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REFERENCES

1. Yamada M, Mari M, Sugimura T: Purification and characterization of small molecular weight myeloperoxidase from human promyelocytic leukemia HL-60 cells. Biochemistry 20:766, 1981

2. Pember SO, Shapira R, Kinkade JM Jr: Multiple forms of myeloperoxidase from human neutrophilic granulocytes: Evidence of differences in compartmentalization, enzymatic activity, and subunit structure. Arch Biochem Biophys 221:391, 1983

3. Morita Y, Inamoto H, Aibara S, Kobayashi T, Hasegawa E: Crystallization and properties of myeloperoxidase from normal human leukocytes. J Biochem 99:761, 1986

4. Yamada M: Myeloperoxidase precursors in human myeloid leukemia HL-60 cells. J Biochem 257:5980, 1982

5. McCandliss R, Sloma A, Pestka S: Isolation and cell-free translation of human interferon mRNA from fibroblasts and leukocytes. Methods Enzymol 79:51, 1981

6. Aviv H, Leder P: Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 69:1408, 1972

7. Gubler U, Hoffman BJ: A simple and very efficient method for generating cDNA libraries. Gene 25:263, 1983

8. Huynh TV, Young RA, Davis RW: Constructing and screening cDNA libraries in λgt10 and λgt11, in Glover DM (ed): DNA Cloning—A Practical Approach, vol 1. Oxford, IRL, 1985, p 49

9. Olmsted JB: Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. J Biol Chem 256:1955, 1981

10. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning—A Laboratory Manual. Cold Spring Harbour Laboratory, Cold Spring Harbor, NY, 1982, p 363

11. Maxam A, Gilbert W: A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560, 1977

12. Nauseef WM, Malech HL: Analysis of the peptide subunits of human neutrophil myeloperoxidase. Blood 67:1504, 1986

13. Feinberg AP, Fogelstein B: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6, 1983

14. Nabeshima Y, Fujii-Kuriyama Y, Muramatsu M, Ogata K: Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature 308:333, 1984

15. Bond BJ, Davidson N: The Drosophila melanogaster actin 5C gene uses two transcription initiation sites and three polyadenylation sites to express multiple mRNA species. Mol Cell Biol 6:2080, 1986

CORRESPONDENCE

MODE OF INHERITANCE OF TYPE II PROTEIN S DEFICIENCY

To the Editor:

Recently, Comp et al[1] have differentiated two types of plasma distribution of protein S in functional protein S deficiency. These authors classified protein S–deficient patients exhibiting normal or mildly reduced total protein S antigen and markedly reduced free protein S as Type I, and those with markedly reduced total protein S antigen as Type II. The inheritance of the Type I defect is clearly autosomal-dominant. However, the pattern of inheritance of the Type II defect is still unknown. Comp et al have suggested that these patients are the offspring of two Type I–deficient individuals and that they inherit an abnormal gene from each parent.

We have studied two siblings with a clinical history of repeated thrombosis since they were 19 and 23 years old. A diagnosis of Type II protein S deficiency could be established from the levels of total protein S antigen of 41% and 31% (normal range 63 to 147) with an absence of free protein S antigen (normal range 52 to 131). Total and free protein S were measured by an electroimmunoassay technique using rabbit antiserum purchased from Diagnostica Stago (Ansieres, France). Free protein S was measured in the supernatant of plasma precipitated with PEG 8.000 as reported elsewhere.[1] The family history of the patients includes repeated episodes of thrombosis in the mother, who died of mesenteric vein thrombosis and the grandfather, who died of multiple pulmonary emboli, suggesting a deficiency in protein S in both cases

The study of the levels of total and free protein S of the father of the siblings deficient in protein S showed normal levels of both proteins: 106% and 99%, respectively. These data do not support the idea put forward by Comp et al regarding the mode of inheritance of Type II protein deficiency, but rather suggest that transmission of the anomaly depends on one or more genes from one of the parents.

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REFERENCE

1. Comp PC, Doray D, Patton D, Esmon CT: An abnormal plasma distribution of protein S occurs in functional protein S deficiency Blood 67:504, 1986

To the Editor.

The formal classification of various types of protein S deficiency will require a firm understanding of the physiologic mechanisms involved. Many of the patients we have tentatively designated as Type I–deficient do have significantly reduced levels of protein S. The Type I group may well be heterogenous in terms of the mechanism of their deficiency. According to the working classification we set out, Type I patients have all or most of their protein S complexed to C4b-binding protein and have low function activity. These patients may have normal or reduced protein S antigenic levels. The two siblings described by Vicente et al probably fall into this Type I group. The two brothers we classify as Type II are unique from the approximately one hundred protein S–deficient patients we have investigated. They are the sons of two Type I individuals. These brothers have little detectable protein S either free in the plasma or in complex with C4b-binding protein. The cross immunoelectrophoretic pattern of these two patients is unique in that no complexed protein S is seen. Crossed immunoelectrophoresis is not a particularly quantitative tool, but does qualitatively demonstrate that these two Type II brothers are significantly different from the other protein S–deficient patients we have examined. Whether or not the two brothers are doubly heterozygous-deficient for traits affecting plasma protein S levels and/or the distribution of protein S between free and complexed forms remains to be seen The fact that they do have some detectable protein S antigen suggests that they are not homozygous-deficient.

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DEOXYHEMOGLOBIN S POLYMER FORMATION IN SICKLE CELLS

To the Editor

We write to call attention to the serious misinterpretation of our work in the recent paper by Billett et al in *Blood* [1] During the last six years we have developed an approach to ascertain deoxyhemoglobin S polymer formation in sickle cells as a function of oxygen saturation, based on ultracentrifugation measurements of the solubility of hemoglobin solutions and nuclear magnetic resonance (NMR) measurements of sickle erythrocytes.[2,3] This approach has allowed us to study quantitatively the effects of intracellular hemoglobin concentration (density distributions of red cells) and composition (% hemoglobin S and % non-S hemoglobins) on intracellular polymerization [4] We have found, in work with Brittenham, that the extent of polymerization correlates very strongly with the severity of anemia and overall vaso-occlusive manifestations among twelve major genetic variants of sickle cell disease.[5] We and our colleagues have used these analyses to explain overall disease severity in SC disease as compared to the AS genotype.[6,7] We have characterized some of the changes in red cell properties in sickle cell anemia with homozygous alpha thalassemia[8] and how these changes may affect polymerization tendency in this condition (Abstract submitted to American Society of Hematology 1986 meeting). This work has also been partially successful in accounting for the anemia and certain aspects of clinical severity (such as chronic leg ulcerations) within a given sickle syndrome,[9] and has recently shown great promise for explaining the effects of varying hemoglobin F levels on overall disease manifestations (Abstract cited above).

In contrast to the assertions of Billett et al, our work does not state or imply that "dense cells" should correlate with the incidence of sickle cell painful crisis, one of the aspects of these diseases that is most complex and difficult to quantitate. Any rigorous test of the hypothesis that a correlation should exist between polymer formation and this, or any other single clinical manifestation in a single sickle cell genotype is, in our opinion, a formidable task. It would require the prospective long-term study of a large number of patients, homogeneous for the known genetic modifiers of sickle cell disease, with generally accepted objective clinical criteria Complete determination of red cell density distributions (by techniques such as preparative Stractan[10] or analytical phthalate ester[11] gradients) and hemoglobin composition would allow calculation of the significant independent variable, polymer formation We hope such studies will eventually be done, perhaps by the Cooperative Study of Sickle Cell Disease, to complement the smaller studies of our colleagues and ourselves

We also hope that the negative results of Billett et al will not obscure the progress that has been made based on the hypothesis that the primary pathophysiological event in sickle cell disease is the reversible intracellular polymerization of hemoglobin S in partially deoxygenated red cells and the resultant rheological abnormalities caused by these cells [3,4,12,13] This hypothesis allows ascertainment of the effects of many of the genetic and cellular modifiers of the polymerization process [4,5] Ultimately, quantitation of other physiological variables, such as possible changes in the red cell membrane, adhesion, blood flow, vascular tone, and tissue oxygenation, will be necessary for a full understanding of disease manifestations in each organ and in each patient.[14,15] This approach, however, is already allowing the gradual development of a quantitative understanding of many of the manifestations of the sickle cell diseases and, most importantly, the establishment of goals for therapies based on manipulation of the sickle red cell, the intracellular hemoglobin, or globin gene expression.[16]

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REFERENCES

1 Billett HH, Kim K, Fabry ME, Nagel RL: The percentage of dense red cells does not predict incidence of sickle cell painful crisis Blood 68 301, 1986

2. Noguchi CT, Torchi DA, Schechter AN· Determination of deoxyhemoglobin S polymer in sickle erythrocytes upon deoxygenation Proc Natl Acad Sci USA 77 5487, 1980

3 Noguchi CT, Schechter AN. The intracellular polymerization of sickle hemoglobin and its relevance to sickle cell disease. Blood 58.1057, 1981

4 Noguchi CT, Schechter AN· Sickle hemoglobin polymerization in solutions and in cells. Annu Rev Biophys Biophys Chem 14·239, 1985

5. Brittenham G, Schechter AN, Noguchi CT. Hemoglobin S polymerization: Primary determinant of the hemolytic and clinical severity of the sickling syndromes. Blood 65:183, 1985

6. Bunn HF, Noguchi CT, Hofrichter HJ, Schechter GP, Schechter AN, Eaton WA· The molecular and cellular pathogenesis of hemoglobin SC disease. Proc Natl Acad Sci USA 79:7629, 1982

7. Noguchi CT. Polymerization in erythrocytes containing S and non-S hemoglobins. Biophys J 45:1153, 1984

8. Noguchi CT, Dover GJ, Rodgers GP, Serjeant GR, Antonarakis SE, Anagnou NP, Higgs DR, Weatherall DJ, Schechter AN: α-Thalassemia changes erythrocyte heterogeneity in sickle cell disease J Clin Invest 75:1632, 1985

9 Rodgers GP, Noguchi CT, Serjeant GR, Schechter AN: The relationship between red cell heterogeneity and various manifestations of sickle cell disease Blood 66 65a, 1985

10 Noguchi CT, Torchia DA, Schechter AN The intracellular polymerization of sickle hemoglobin Effects of cell heterogeneity. J Clin Invest 72:846, 1983

11. Rodgers GP, Schechter AN, Noguchi CT. Cell heterogeneity in sickle cell disease: Quantitation of the erythrocyte density profile. J Lab Clin Med 106 30, 1985

12 Harris JW, Kellermeyer RW: The Red Cell: Production, Metabolism, Destruction. Normal and Abnormal Harvard University, Cambridge, MA, 1970, p 168

13 Hofrichter J, Ross PD, Eaton WA: A physical description of hemoglobin S gelation, in Hercules JI, Cottam GL, Waterman MR, Schechter AN (eds). Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease. DHEW Publ No (NIH) 76-1007, 1976, p 185

14. Rodgers GP, Schechter AN, Noguchi CT, Klein HG, Nienhuis AW, Bonner RF Periodic microcirculatory flow in patients with sickle cell disease. N Engl J Med 311:1534, 1984

15. Rodgers GP, Noguchi CT, Nienhuis AW, Schechter AN: The development of quantitatives indices of disease severity in the sickle cell syndromes, in Beuzard Y, Charache S, Rosa J (eds) Approaches to the Therapy of Sickle Cell Anemia. Les Editions INSERM, Paris, 1986, p 487

16 Schechter AN, Noguchi CT, Rodgers GP Sickle cell anemia, in Stamatoyannopoulos G, Nienhuis AW, Leder P, Majerus PW (eds). Molecular Basis of Blood Diseases Philadelphia, Saunders, 1987, p 179

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"Incorporation of Circulating Proteins into Megakaryocyte and Platelet Granules"
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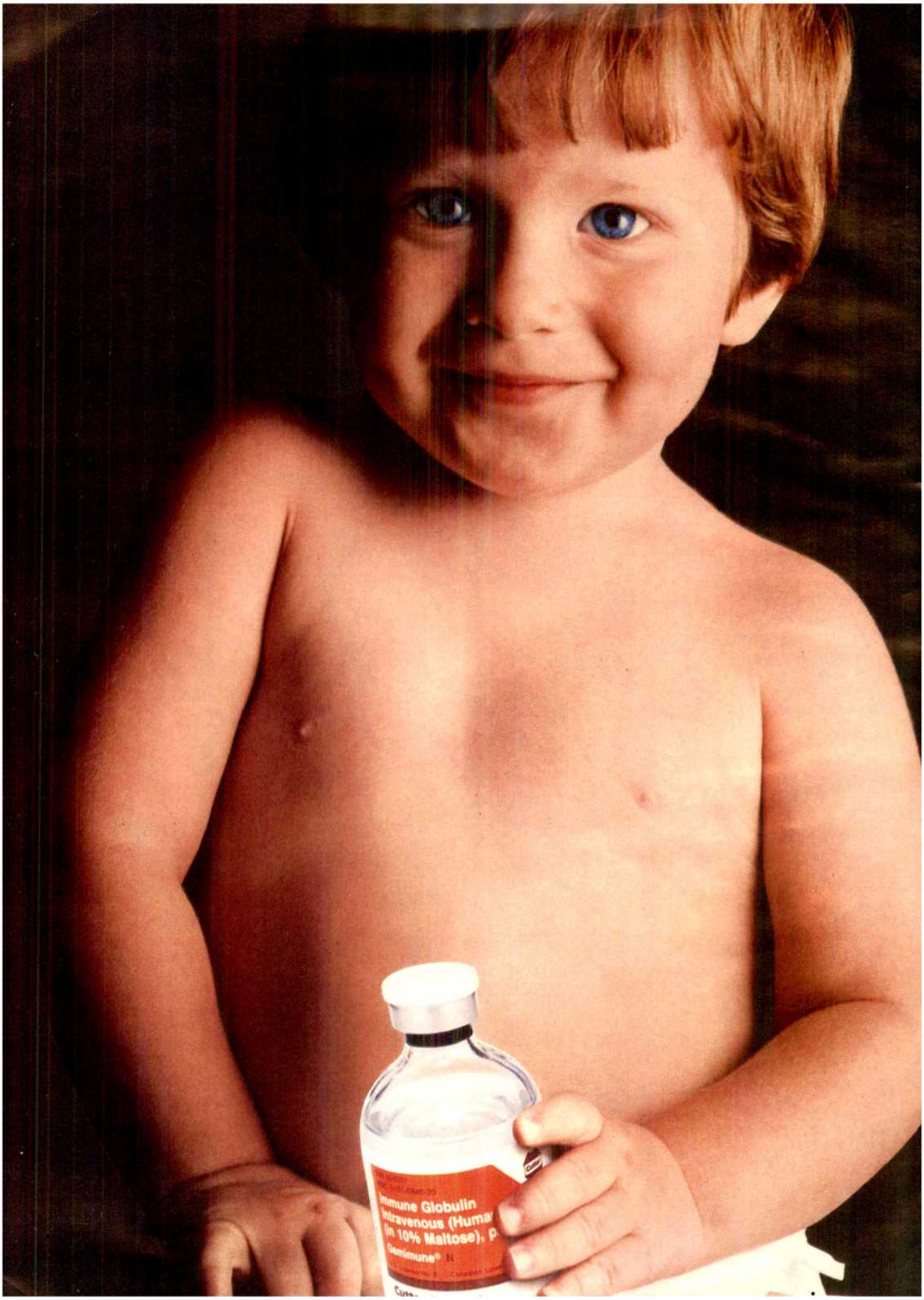
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*The Cohn-Oncley process used to produce GAMIMUNE® N has been challenged *in vitro* with a virus spike of 1 x 10^5 of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, GAMIMUNE® N undergoes an incubation step as a final container which has been shown to effect an additional 1 x 10^3 to 1 x 10^4 reduction of HTLV-III.[2] In a separate study of intravenous immune globulins conducted by the Food and Drug Administration, an *in vitro* challenge at each of the six Cohn-Oncley fractionation steps using HTLV-III demonstrated a cumulative reduction of greater than 1 x 10^{15} infectious units.[3]

Please see following page for brief summary of prescribing information.

GAMIMUNE® N
Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The in vivo half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1–5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100–200 mg/kg (2–4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. GAMIMUNE® N Package Insert. **2.** Mitra G, Wong MF, Mosen MM, et al: Elimination of infectious retrovirus during preparation of immunoglobulins. Submitted for publication in Transfusion. **3.** Wells MA, Wittek AE, Epstein JS, et al: Inactivation and partition of human T-cell lymphotropic virus, type III, during ethanol fractionation of plasma. Transfusion 1986; 26(2): 210–213.

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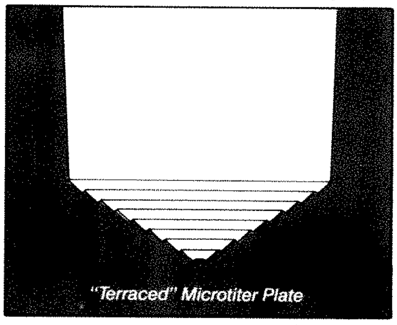
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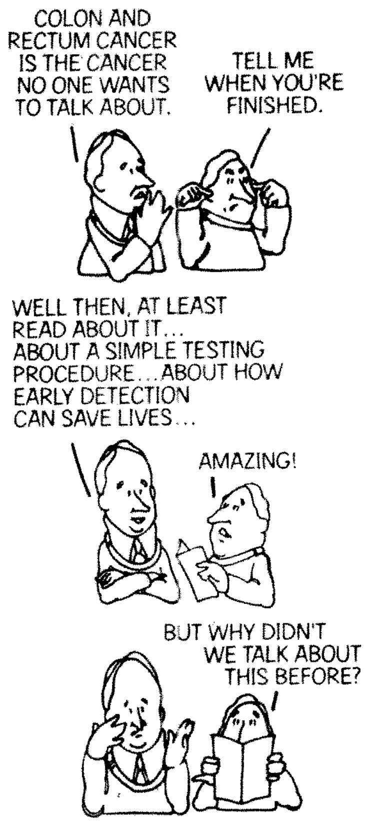
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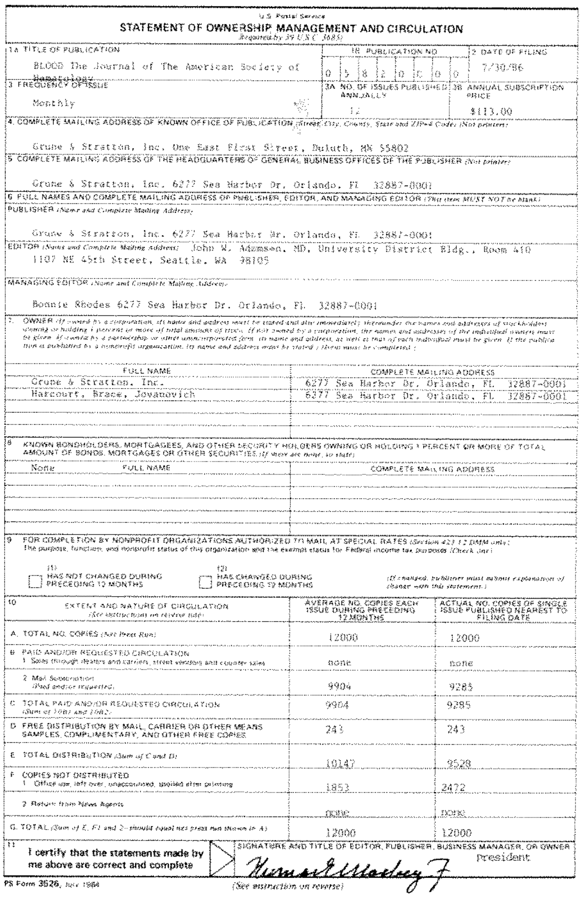

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DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD, contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from donors found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III Antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum level is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea were occasionally observed during our clinical trials. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS).

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring serum IgG levels before each dose, to insure that the do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/k per Hr be used. If infusion at this rate causes th patient no distress, the administration rate may b gradually increased but should not exceed 4 mL/k per Hr.

A rate of administration which is too rapid ma cause flushing and changes in pulse rate and bloo pressure. Slowing or stopping the infusion usuall allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human GAMMAGARD should be administered as soon aft reconstitution as possible. Administration should b gin not more than 2 hours after reconstitution.

The reconstituted material should be at roo temperature during administration.

Parenteral drug products should be inspecte visually for particulate matter and discoloration pri to administration, whenever solution and contain permit.

Follow directions for use printed on the administr tion set container. If a Hyland set is not used, mak sure the administration set contains an adequate filte

How Supplied

Immune Globulin Intravenous (Human GAMMAGARD is supplied in either 2.5 g or 5.0 g sing use vials. Each vial of Immune Globulin Intraveno (Human), GAMMAGARD is furnished with a suitab volume of Sterile Water for Injection, USP, a transf device and an administration set which contains integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human GAMMAGARD should be stored under ordina refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing shou be avoided to prevent the diluent bottle from breakin

REFERENCES

1. Unpublished data in files of Travenol Laboratorie Inc.
2. Waldman TA, Storber W: Metabolism of immun globulins. *Prog Allergy 13:* 1-110, 1969.
3. Morell A, Riesen W: Structure, function and cat bolism of immunoglobulins in *Immunohemotheraj* Nydegger UE (ed), London, Academic Press, 198 pp 17-26.
4. Stiehm ER: Standard and special human immu serum globulins as therapeutic agents. *Pediatri* 63:301-319, 1979.
5. Buckley RH: Immunoglobulin replacement therap Indications and contraindications for use and variab IgG levels achieved in *Immunoglobulins: Characte istics and Use of Intravenous Preparations.* Alvi BM, Finlayson JS (eds), Washington, D.C., U.S. D partment of Health and Human Services, 1979, pp3-
6. Burks AW, Sampson HA, Buckley RH: Anaphylac reactions following gammaglobulin administration patients with hypogammaglobulinemia: Detection IgE antibodies to IgA. Submitted for publicatio

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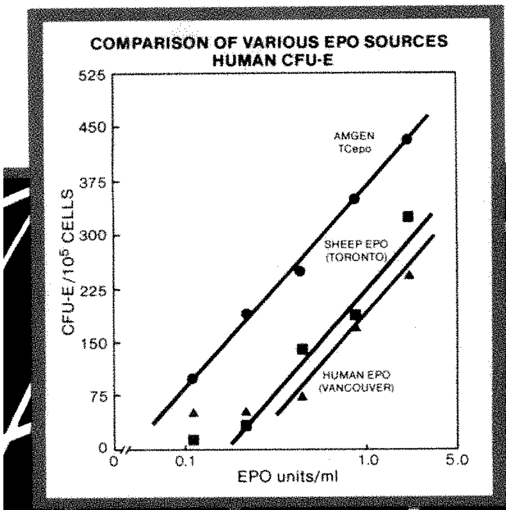

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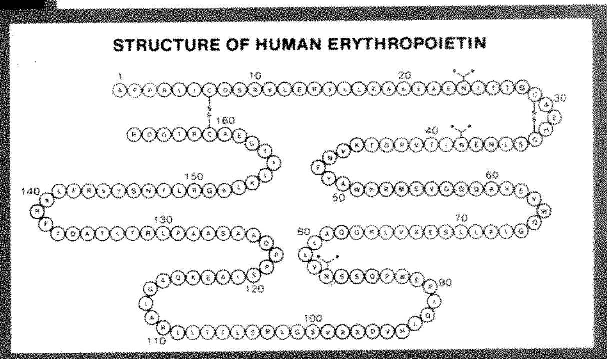

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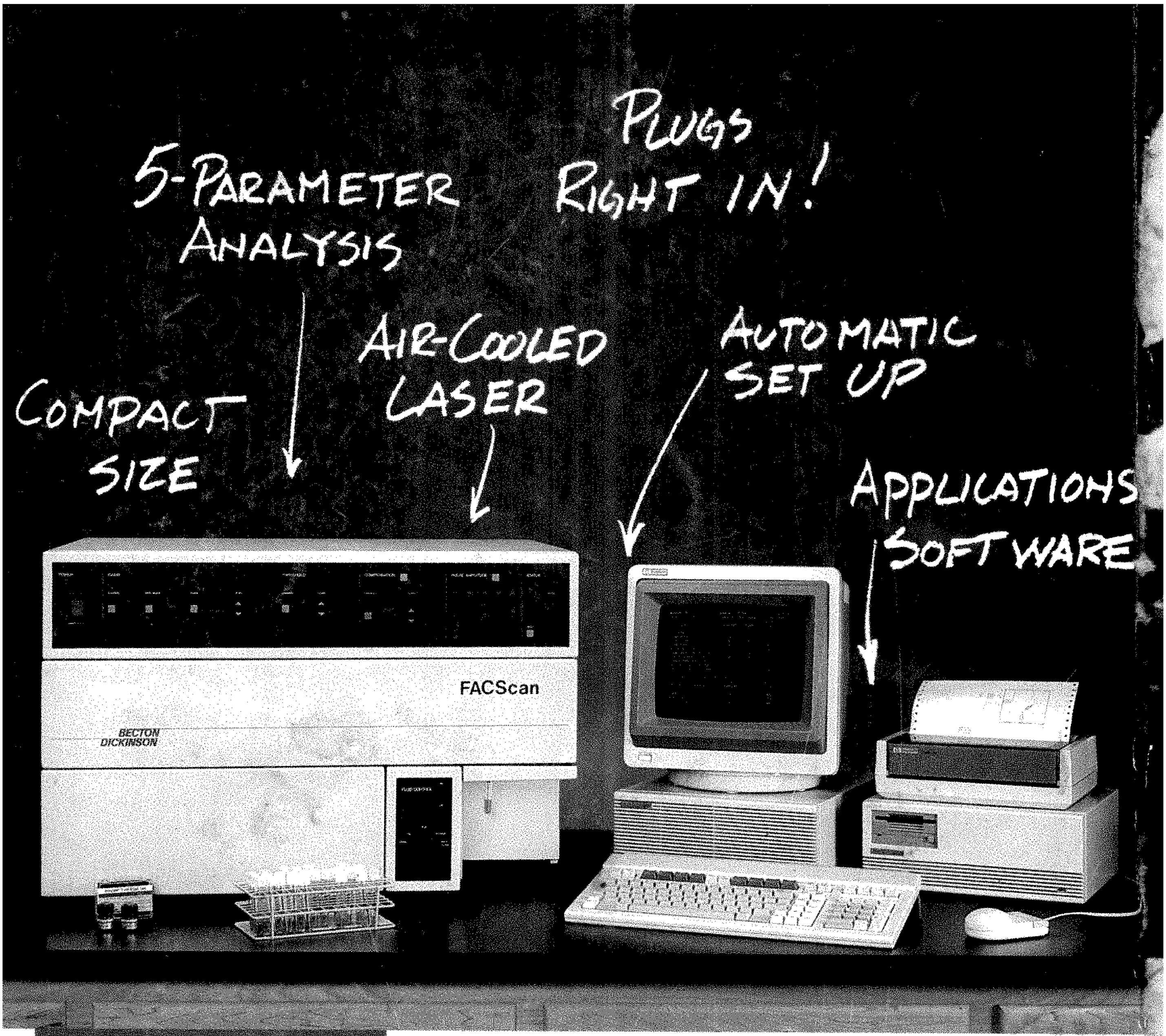

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